The TOR Pathway, a Central Relay Linking Cell Growth and Cell Wall Dynamics in *Arabidopsis thaliana*

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1.1 Summary

Plant cells are surrounded by a rigid wall that confers protection and shape but the cell wall also limits cell growth. Therefore, controlled cell wall expansion is necessary to allow cells to grow. However, it remains elusive how plant cells manage to orchestrate growth and the essential cell wall adaptions. To date, a number of proteins such as receptor-like kinases have been identified that relay information of the cell wall to the cytoplasm but the intracellular targets still remain to be elucidated. The TOR pathway is a central controller of growth in all eukaryotes. It senses environmental conditions and influences cell growth and shape. This makes TOR an ideal candidate for combining the regulation of cell growth with the necessary changes in the cell wall structure. In *Arabidopsis thaliana*, ROL5 has been identified to influence cell wall structures leading to suppression of the *lrx1* root hair phenotype. This phenotype is characterized by aberrant root hair formation, and bulbous-like structures at the root hair base that frequently burst. LRX proteins are not only structural cell wall components but also have a regulatory function during cell wall formation. In this thesis, ROL5 was also identified to influence TOR signaling. Thus, first evidence is provided that TOR is indeed involved in cell growth and in the modification of the cell wall. This made it very interesting to examine how ROL5 fulfils these functions. To this end, several approaches were followed. These included the search for potential ROL5 interacting proteins with yeast two-hybrid, and protein co-purification experiments. In the course of this, the small GTPase ROP6 was identified as an interactor. Small GTPases are influencing polar cell growth, and have been shown to be important for cell wall integrity maintenance and TOR signaling in yeast. This made ROP6 a candidate for connecting TOR and ROL5.

ROL5 is not only influencing TOR and cell wall development but also revealed to be important for the thiolation of tRNAs. This process is not necessary for cell viability but improves the translational efficiency. In humans, the severe condition myoclonus epilepsy is caused when tRNA thiolation is impaired. Patients exhibit severe disorders in mitochondrial translation. In this work, we show that

ROL5 apparently has dual functions in tRNA modification and TOR signaling. The investigation of the ROL5 protein interaction network led to the identification of further proteins which are known to be involved in tRNA thiolation in yeast. Subsequently, these Arabidopsis proteins were tested for their ability to function in tRNA thiolation. This revealed that the process of tRNA thiolation is quite conserved in Arabidopsis and yeast.

1.2 Zusammenfassung

Pflanzenzellen erhalten Schutz und ihre Form durch die sie umgebende, stark widerstandsfähige Zellwand. Andererseits verhindert die Zellwand dadurch aber auch das Zellwachstum. Damit Zellwachstum möglich wird, muss diese Barriere regulierte, strukturelle Veränderungen durchlaufen können um eine Ausdehnung zu ermöglichen. Das TOR (Target of Rapamycin) Netzwerk gleicht einer Schaltzentrale, die Umwelteinflüsse wahrnimmt, verarbeitet und entsprechend Zellwachstum ermöglicht oder unterbindet. Bis heute ist unklar, wie Zellen das Zellwachstum auf der einen und die nötigen Veränderungen in der Zellwand auf der anderen Seite aufeinander abstimmen. Das TOR Netzwerk ist ein idealer Kandidat um diese Aufgabe zu realisieren. In *Arabidopsis thaliana* konnte gezeigt werden, dass das Protein ROL5 die Zellwandstruktur beeinflusst. Dies wurde ersichtlich da eine Mutation im *ROL5* Gen zur Unterdrückung des *lrx1* Wurzelhaarphänotyps führt. *lrx1* Mutanten zeigen eine fehlerhafte Wurzelhaarbildung, welche durch eine geschwächte Zellwand hervorgerufen wird. Das *LRX1* Protein hat nicht nur eine strukturelle, sondern auch eine regulatorische Funktion in der Pflanzenzellwandentwicklung. In dieser Arbeit konnte gezeigt werden, dass das ROL5 Protein einen Einfluss auf das TOR Netzwerk hat. Dies ist der erste Hinweis darauf, dass TOR in der Tat nicht nur das Zellwachstum beeinflusst sondern auch die gezielte Modifikation der Zellwandstruktur ermöglicht. Mit verschiedenen Methoden wurde daraufhin untersucht wie ROL5 dies bewerkstelligt.

Unter anderem wurden mehrere physische Interaktoren von ROL5 ermittelt. Darunter auch das kleine G-Protein ROP6. Diese Klasse von Proteinen ist notwendig für das polare Zellwachstum und für die Aufrechterhaltung der Integrität der Zellwand. Somit ist ROP6 ein idealer Kandidat um die Verbindung zwischen ROL5 und TOR herzustellen.

ROL5 beeinflusst aber nicht nur das TOR Netzwerk sondern auch die Modifikation von tRNAs, ein Prozess der die Proteinbiosynthese effizienter ablaufen lässt. Ein Fehler in diesem Prozess löst bei Patienten die Myoklonus Epilepsie aus. In dieser Arbeit zeigen wir, dass die Thiolierung von tRNAs und die Beeinflussung von TOR zwei unabhängige Funktionen von ROL5 darstellt. Zusätzlich wurden bei der Untersuchung des ROL5 Interaktions-Netzwerks unter anderem auch mehrere Proteine identifiziert deren Homologe aus Hefe bekanntermassen für die Modifikation von tRNAs zuständig sind. Im Folgenden wurden diese Arabidopsis Proteine auf eine mögliche Funktion bei der Modifikation von tRNAs hin untersucht. Hierbei konnten wir eine starke Konservierung dieses Proteinnetzwerks zwischen Arabidopsis und Hefe feststellen.

2 Introduction

2.1 Properties of the plant cell wall

Plant cell walls have to fulfil several very diverse tasks like conferring mechanical strength and protection to the cell, as well as allowing cell-cohesion and -communication. But cells are also dynamic structures underlying growth and developmental processes. Therefore, cell walls nowadays are regarded not as static shells but as an extension of the cytoplasm. In addition, cell walls are also reacting upon alterations in the environment like osmotic stress or pathogen attack with changes in their composition (Carpita, and Gibeaut, 1993). Since there is a vast ecological diversity in plants this is also reflected by a huge number of different cell forms and thereby different types of cell walls fitting the particular needs (Cassab, 1998). Primary cell walls have a fibreglass-like structure, mainly composed of cellulose microfibrills which are embedded in a matrix of pectins and hemicelluloses which are complex polysaccharides (Figure 1) (Cosgrove, 2005).

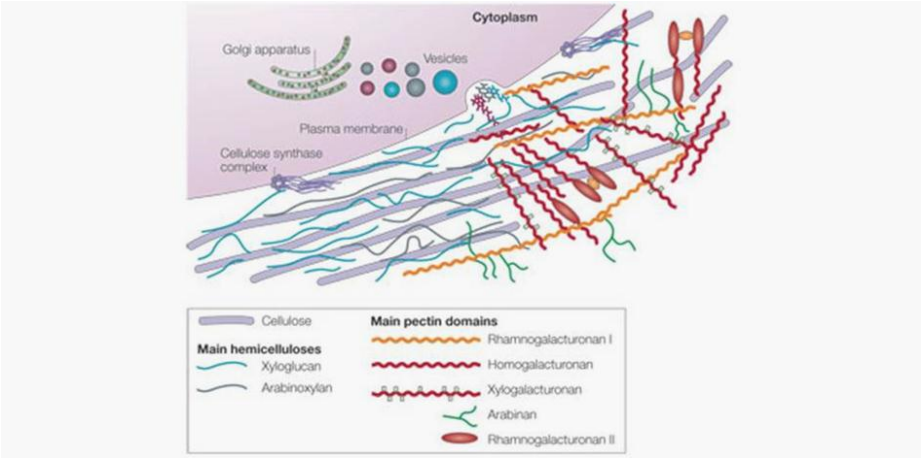

Figure 1 Structure of the primary cell wall. The cellulose microfibrills are embedded in a matrix of pectins and hemicelluloses. But the cell wall is not a rigid shell; instead it is more seen to be an extension of the cytoplasm (Figure from Cosgrove, 2005).

Other important components are cell wall proteins which are relatively abundant among plants. These proteins are very repetitive, and often glycosylated. The cell wall proteins can be divided into

three groups: the hydroxyproline-rich glycoproteins (HRGPs), the proline-rich proteins (PRPs), and the glycine-rich proteins (GRPs). Since the abundance of these proteins differs, it is assumed that they confer particular functions to different cell types (Cassab, 1998).

2.1.2 The polysaccharide components of the plant cell wall

Cellulose is assembled in form of insoluble microfibrills that are typically composed of approximately 36 hydrogen-bonded chains containing 500 to 14,000 ß-1,4-linked glucose molecules each. Cellulose microfibrils are the main component of plant cell walls. The transversally oriented layers of microfibrils allow the cells to withstand the turgor pressure. The cellulose synthesis complex (CelS) which harbours the ß-glycosyl transferase activity is composed of CesA protein isoforms. The family of *CesA* genes consists of 6 genes (*AtCesA1, 3, 4, 6, 7, 8)*. The corresponding mutants have been shown to be cellulose deficient (Doblin et al., 2002). At least three of these AtCesA isoforms are required in one cell for normal cellulose synthesis (Robert et al., 2004). In Arabidopsis, mosses, ferns, algae, and vascular plants, CesA subunits form hexagonal structures with six-fold symmetry (rosettes) and localize to the plasma membrane. Six CesA proteins form one rosette subunit, and six rosette subunits form one rosette. These rosettes do not consist of the same but of a mixture of CesA isoforms. Hence, all together one rosette is able to produce 36 parallel cellulose microfibrills. These rosettes are assembled in the Golgi, and subsequently transported to the membrane.

Hemicelluloses are cell wall polysaccharides that can be solubilised by aqueous alkali. They have been classed as cross-linking glycans and are able to cross-link cellulose microfibrills. Hemicelluloses consist of several polysaccharide groups with the most important ones being xyloglucans, xylans, mannans, and mixed-linkage glucans (Lee et al., 2011). Xyloglycan is the most abundant hemicellulose in dicot primary walls, and consists of a β-1,4-glucan backbone with 1,6-α-xylosyl residues. Xyloglycan is thought to contribute to the mechanical properties of the cell wall by acting as a tether between cellulose microfibrills (Cafall et al., 2009; Hayashi and Kaida, 2011). Xylans are

comprised of linear chains of β-(1,4)-D-Xylp residues, and can be found as arabinoxylan (AX), glucuronoarabinoxylan (GAX), glucuronoxylan (GX), or the unsubstituted homoxylan. Finally, mannans are structural and important storage polysaccharides of the cell wall (Cafall et al., 2009).

Pectins represent a group of very complex polysaccharides which are components of the primary plant cell wall and contain 1,4-linked -α-D-galacturonic residues. Pectin consists of rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII), and homogalacturonan (HG) which differ in their sugar backbone structure. The RGI backbone is composed of the repeating disaccharide α-D-galacturonic acid-rhamnose. The side chains are mainly composed of linear and branched α-1-arabinose and or β-D-galactose residues. RGII instead is composed of an HG backbone and contains several conserved side chains formed by different sugars. HG is a homopolymer of 1,4-galacturonic acid which is synthesized as methylester (Ridley et al., 2001; Willats et al., 2001). The stiffness of the cell wall can be altered by enzymatic action on pectic components (Willats et al., 2001; Micheli, 2001). Pectins may be demethylated by pectin methylesterase (PME) (Moustacas et al., 1991). PME is able to change the stiffness of the cell wall by creating free carboxyl groups which interact with Ca^{2+}, thereby creating a pectate gel. The cell wall-modifying activity of PME has been shown to be involved in processes like fruit maturation, microsporogenesis, and pollen tube growth, cambial cell differentiation, seed germination, and hypocotyl elongation (Micheli, 2001).

2.1.3 Structural cell wall proteins

Structural cell wall proteins may be divided into three major classes, the hydroxyproline-rich glycoproteins (HRGPs), which contain the extensins and the arabinogalactan proteins as major classes, the proline-rich proteins, and the glycine-rich proteins (Cosgrove, 2005). Most of these proteins, as their name suggests, are cross-linked to the cell wall, have a structural task, and some of them are thought to be involved in cell-cell interaction (Cassab, 1998). For this work, I like to focus on the group of extensins.

Extensins

Extensins are the best studied structural cell wall proteins. Characteristically they contain a Ser-(Hyp) $_4$ motif which seems to have a structural function (Cassab, 1998). All are rich in hydroxyproline (Hyp), and serine (Ser), and most of the Hyp residues are glycosylated with one to four arabinosyl residues, while Ser residues are frequently glycosylated with a single Gal unit. The connection to the cell wall is supposed to be established by a covalent link (Cooper et al, 1983). Various experiments suggest that insolubilisation of extensins in the cell wall is established by oxidative cross-linking via Tyr residues (Fry, 1982; Brady et al., 1998; Ringli et al., 2001; Held et al., 2004). Regarding their function, it is thought that extensins are structural cell wall components which might also play a role in development, wound healing, and plant defense since it has been shown that extensin accumulation is associated with cessation of cell growth or with plant pathogen defence (Sadava et al, 1973; Brisson et al., 1994; Shirsat et al, 1996 ; Cassab, 1998; Hall and Cannon, 2002; Zhang et al., 2008).

LRR-extensins

LRR-extensins are chimeric proteins composed of a leucine-rich repeat domain and an extensin-like domain. LRR domains are usually involved in protein-protein interaction and often represent the extra cellular domain of plant signaling proteins (Wang et al., 2010). This makes LRX proteins good candidates for being regulators of cell wall development. While the LRR domain consists of 9 repetitions of 23 up to 25 amino acids, the extensin-like domain is composed of diverse variations of the $(Ser\text{-}Hyp_4)_n$ backbone. In Arabidopsis 11 putative *LRX* genes were identified which can be divided into two classes with expression in vegetative and reproductive tissues, respectively. *AtLRX1*, At*LRX2*, and *AtLRX6* are specifically expressed in roots whereas the remaining *AtLRX* genes are expressed in roots and shoots or in the pollen (Baumberger et al., 2003). Via analysis of the respective mutants, *AtLRX1* and At*LRX2* were linked to cell wall formation and assembly in root hairs (Baumberger et al., 2001; 2003).

This complex network of cell wall components underlies frequent changes during cell morphogenesis or in response to biotic or abiotic stress. Therefore, the integrity of the cell wall has always to be controlled and maintained, which requires elaborate sensing mechanisms.

2.2 Plant cell wall integrity sensing

Plant cells need to be able to adapt and maintain the integrity of their cell wall when changes in cell morphology occur, and in response to biotic or abiotic stress. The underlying mechanism of this regulatory process is poorly understood. In yeast, cell wall integrity (CWI) is sensed by specialized membrane-spanning receptors and by cross-talk to other stress response pathways. These mechanisms monitor diverse parameters like membrane stretch, cell wall damage, osmotic stress, and oxidative stress. Also in plants there is evidence for the existence of similar CWI sensing systems (Hamann, 2011). Two mechano-sensitive receptors have been described which affect mechano-perception in protoplasts derived from Arabidopsis root cells. These are namely MCA1, and MCA2. Both proteins are stretch-activated calcium channels. MCA1 has been shown to be important for cell wall defective response signaling. The *mca1* mutant exhibits root growth defects, a decreased calcium influx in root cells upon mechano-stimulation, and less ectopic lignin is deposited upon cellulose biosynthesis inhibition (Denness et al., 2011).

Oligogalacturonides (OGAs) are produced by cell wall degrading enzymes from HG and have been shown to influence several cellular processes, including cell wall reinforcement (Hahn et al., 1981; Denoux et al., 2008; Ferrari et al., 2008). OGAs are supposed to be ligands of Wall Associated Kinase 1 (WAK1) which signals through its intracellular domain that is structurally similar to elongation factor Tu receptor (EFR) kinase (Brutus et al., 2010). In Arabidopsis a huge number of receptor like kinases (RLK) have been identified. RLK THESEUS1 (THE1) has been shown to be involved in signaling when cellulose synthesis is impaired and to adapt cell wall development upon changes or irregularities. Therefore, THE1 might act as a cell wall maintenance sensor (Hematy et al., 2007;

Ringli, 2010). Besides THE1 also the RLKs HERCULES1 and FERONIA have been linked to cell wall integrity maintenance (Hamann, 2011).

The information produced by the CWI sensing mechanisms have to be processed and relayed to induce the necessary changes in plant metabolism and subsequently in cell wall structure. This implies the necessity of a central controlling mechanism. The Target of Rapamycin (TOR) is a central controller of growth and metabolism, and has also been shown to influence cell wall composition (Leiber et al., 2010). This makes TOR a promising candidate for integrating the signals produced by the CWI mechanisms and inducing the necessary adaptions. This is corroborated by the fact that CWI is indeed linked to TOR signaling in yeast (Levin, 2005; Fuchs et al., 2009; Tsao et al., 2009).

2.3 TOR signaling

First evidence for existence of the TOR signaling network was obtained by observation that mutations in TOR1 and TOR2 in *Saccharomyces cerevisiae* led to resistance to the growth inhibitory action of rapamycin (Heitman et al., 1991). Rapamycin is a macrocyclic lactone and was originally isolated from *Streptomyces hygroscopicus* on Easter Island. TOR represents a central switch, promoting or arresting cell growth in response to environmental stimuli. Cell growth-promoting stimuli are the availability of nutrients and growth factors, while stress arrests growth (Wullschleger et al., 2006).

TOR proteins of eukaryotes have an approximate size of about 280kDa, and belong to the group of phosphatidylinositol kinase-related kinases (PIKK) (Wullschleger et al., 2006). Depending on the species, the TOR kinase is complexing with several proteins, forming two complexes with different biological activities. Most information about the nature of these complexes is available in *Saccharomyces cerevisiae* and in mammals. In humans, TORC1 is formed by mTOR, RAPTOR, and mLST8. TORC2 is known to consist of mTOR as well as RICTOR and mLST8. Only TORC1 signaling is known to be sensitive to rapamycin which requires the interaction with the peptidyl-prolyl-cis/trans-

isomerase FKBP12, and the FRB domain of TOR. In contrast, TORC2 signaling is rapamycin insensitive (Loewith et al., 2002; Wullschleger et al., 2006). TORC1 controls several cell growth-related anabolic and catabolic processes including protein synthesis and metabolism, ribosome biogenesis, transcription of mRNAs and tRNAs, and autophagy. In contrast, TORC2 determines where the cell grows by influencing the cellular cytoskeleton. In Arabidopsis, little is known about the formation of TORC1 and TORC2. The review in the next chapter summarizes our current understanding of the TOR pathway in plants. In addition, the functional LST8 homolog has recently been described in Arabidopsis (Moreau et al., 2012).

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Plant TOR signaling components

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Keywords: Target of Rapamycin, TOR signaling, plants, cell growth, cell wall, ROL5, nutrient sensing

Cell growth is a process that needs to be tightly regulated. Cells must be able to sense environmental factors like nutrient abundance, the energy level or stress signals and coordinate growth accordingly. The Target Of Rapamycin (TOR) pathway is a major controller of growth-related processes in all eukaryotes. If environmental conditions are favorable, the TOR pathway promotes cell and organ growth and restrains catabolic processes like autophagy. Rapamycin is a specific inhibitor of the TOR kinase and acts as a potent inhibitor of TOR signaling. As a consequence, interfering with TOR signaling has a strong impact on plant development. This review summarizes the progress in the understanding of the biological significance and the functional analysis of the TOR pathway in plants.

The TOR kinase acts as a central component of TOR signaling and modifies several downstream proteins by phosphorylation. TOR is part of two distinct multi protein complexes, namely TORC1 and TORC2, which are controlling diverse cellular processes such as autophagy, protein translation, ribosome biogenesis, and actin dynamics.[1] Mitochondrial oxidative function, which impacts aging processes, and the production of reactive oxygen species (ROS) are also strongly influenced by TOR signaling.[2] ROS can have cytotoxic effects but also possess a very important function as signaling molecules in diverse cellular processes. In plants, these molecules have been shown to be important for pathogen defense, polar cell growth, and the remodeling of the cell wall.[3]

The name of the TOR kinase and the entire pathway describes a characteristic property—the specific and effective inhibition by rapamycin, a macrocyclic lactone of bacterial origin. This sensitivity makes rapamycin a drug with very interesting properties for clinical and basic research.[4-6] Its potential is already used in tumor treatment, cardiology, transplantation medicine and treatment of neuronal diseases.[7-9]

Structure of the TOR Protein

The TOR protein belongs to the family of PIKK (phosphatidylinositol kinase-related kinases) which represent a group of conserved serine/threonine kinases. In addition to the kinase domain, the TOR protein possesses further distinct domains. In the N-terminal region, TOR consists of up to 20 tandem HEAT repeats (Huntingtin, elongation factor 3 [**E**F3], protein phosphatase 2A [PP2**A**], yeast PI3-kinase **T**OR1), followed by the FAT domain (FRAP/ATM/TRRAP), the FRB domain, the kinase domain and the FATC domain (**Fig. 1**). The latter four domains are found in all PIKKs and thus seem important for the activity of this class of kinases. The HEAT repeats have been shown to mediate protein-protein interactions and are found in several cytoplasmic proteins including the four giving rise to the acronym.[10] The inhibition of TOR by rapamycin requires the formation of a ternary complex of rapamycin, the peptidyl-prolyl cis/trans isomerase FKBP12, and the FRB (FKBP12 rapamycin binding) domain.[1,4,11] The redox state of the FATC domain seems to impact the degradation of TOR.[12] Yet, Ren and workers have shown that in *Arabidopsis thaliana*, the FATC domain is not essential for TOR function.[13] For a detailed structural analysis of TOR, see Knutson (2010).[10]

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Plant TOR Proteins

TOR kinases from very diverse eukaryotic species show a high degree of conservation in the kinase, FATC and the FRB domain but only to a limited extend in the number of HEAT repeats. As for most eukaryotes, the plant model species *Arabidopsis thaliana* possesses a single *TOR* gene coding for a protein of approximately 280 kDa.[14] Maize (*Zea mays*) is the only other plant for which the TOR kinase has been described. As for Arabidopsis, the maize TOR protein is encoded by one gene and is also comparable in size to the Arabidopsis TOR protein.[15] The closest homologs of the Arabidopsis TOR are the TOR proteins of *Populus trichocarpa* (identity: 82%, similarity: 89%) and the TOR protein isoforms one (identity: 80%, similarity: 88%) and two (identity: 79%, similarity: 87%) of *Vitis vinifera*. In all these plant proteins, the general domain structure as well as the protein sequence is well conserved. The phylogenetic tree of TOR proteins from different species perfectly reflects phylogenetic relationships of the species. Four main groups are visible corresponding to the animal kingdom, fungi, algae and higher plants, respectively (**Fig. 2**). This strong conservation of TOR proteins throughout the species points out the general importance of this kinase and, consequently, the entire TOR pathway.

Sensitivity to Rapamycin

TOR activity can be inhibited by direct interaction of rapamycin with the cis/trans isomerase FKBP12 and the FRB domain of the TOR protein.[5] In animals, TOR is sensitive to rapamycin. Also

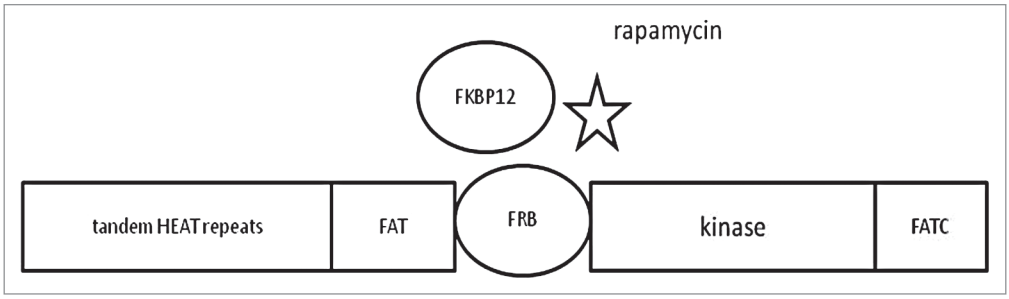

Figure 1. Structure of the TOR protein. The TOR protein belongs to the family of PIKK (phosphatidylinositol kinase-related kinases). In the N-terminal region, TOR proteins possess up to 20 tandem HEAT repeats which have been shown to mediate protein-protein interactions. The catalytic domain is flanked at the N-terminus by the FAT (FRAP/ATM/TRRAP) and the FRB domain, the latter being the binding site of the TOR-inhibiting drug rapamycin and the peptidyl-prolyl cis/trans isomerase FKBP12. C-terminal of the kinase domain is the FATC domain which seems to influence TOR turnover rate in response to the redox state of the cell.

for maize and the unicellular algae *Chlamydomonas reinhardtii,* rapamycin sensitivity was demonstrated.[16,17] Some land plants, however, lost this sensitivity. Among these plants are Arabidopsis and *Vicia faba*.[18] In Arabidopsis the sensitivity to rapamycin can be restored by the expression of *FKBP12* of *S. cerevisiae*. These lines display a reduction in primary root lengths, epidermal cell lengths, less polysome accumulation and an overall reduction of growth after treatment with rapamycin.[19,20]

Functional Importance of the TOR Kinase in Plants

Both in plants and animals, TOR exerts a very general function in anabolic and catabolic processes as described above. In Arabidopsis, a promotor-*GUS* fusion construct revealed *TOR* expression throughout early development in the endosperm, the embryo and the chalazal proliferating tissue. After the early globular stage, *TOR* is no longer expressed in the endosperm but persists in the embryo up to the heart and torpedo stages. In both the seedling and adult plant, *TOR* expression can be detected to a high level in the primary meristems. This suggests that *TOR* expression in Arabidopsis is predominant in zones where cell proliferation is coupled to cytosolic growth, which would be in contrast to mammalian cells and Drosophila were *TOR* expression occurs in all tissues.[14,21,22] Microarray data, however, suggest that a basal level of *TOR* expression is found in all Arabidopsis tissues.[23] In maize, *TOR* expression has been shown to begin during germination at approximately 12 h and increases to the highest level at 48 h. Also, *TOR* RNA has been shown to be present in in all tissues of 13 d-old seedlings at almost the same level, regardless of their developmental stage.[15]

Elucidating TOR function in Arabidopsis by mutations is hampered by the fact that a *TOR* knockout mutant shows an embryo-lethal phenotype and arrests endosperm and embryo development at a premature stage.[14] Thus, a further functional characterization effort was performed using an ethanol-inducible RNAi system. After *TOR* silencing was induced, the treated plants showed several severe growth defects. On the one hand, they almost completely stopped growth of existing leaves and on the other hand, the silenced plants showed symptoms which are usually linked to plant senescence such as early yellowing due to chlorophyll breakdown, accumulation of soluble sugars to a very high degree in the leaves, and a 2–3 fold higher glutamine synthetase and glutamate dehydrogenase activity.[24] Under stress conditions or during senescence, plant cells are recycling cytoplasmic content. This process is called autophagy and has been shown to be influenced by TOR signaling.[25] In Arabidopsis it could be shown that RNAi-*TOR* plants had constitutive autophagy and that some genes required for autophagy were upregulated.[26] Silencing of *TOR* has also an impact on the efficiency in mRNA translation, reflected by a reduction in the abundance of high molecular weight polysomes and a decrease in the amount of soluble protein. A higher level of *TOR* mRNA, instead, causes enhanced organ growth and a higher seed production.[24] In Arabidopsis, plants exposed to osmotic stress develop shorter primary roots. If *TOR* is constitutively expressed in Arabidopsis, this effect is alleviated.[24] These findings suggest that also in plants, TOR is influencing anabolic and catabolic growth processes as well as aging and nutrient recycling and seems to render plants more stress-resistant.

TOR-Binding Proteins

In mammals and yeast, TOR forms two multiprotein complexes TORC1 and TORC2, which contain at least TOR, mLST8, RAPTOR (regulatory associated protein of TOR) and TOR, mLST8, RICTOR, respectively.[1] RAPTOR functions in recruiting TOR substrate proteins and Arabidopsis encodes two RAPTOR proteins, *RAPTOR1A* and *RAPTOR1B*.[27] Homozygous knockout mutants of *raptor1a* do not show any visible mutant phenotype whereas homozygous *raptor1b* mutants exhibit a strong growth phenotype with slower growing roots which are thicker than the wild-type, coiled, and densely covered with root hairs. The phenotype appears to be caused by reduced meristematic activity, which is even further reduced in a *raptor1a raptor1b* double mutant.[28,29] In summary, the disturbance of the Arabidopsis TORC1 multiprotein complex leads to growth defects, emphasizing the importance of the TOR pathway as a growth control mechanism also in plants. Additional components of TORC1 as well as those of TORC2 remain to be analyzed.

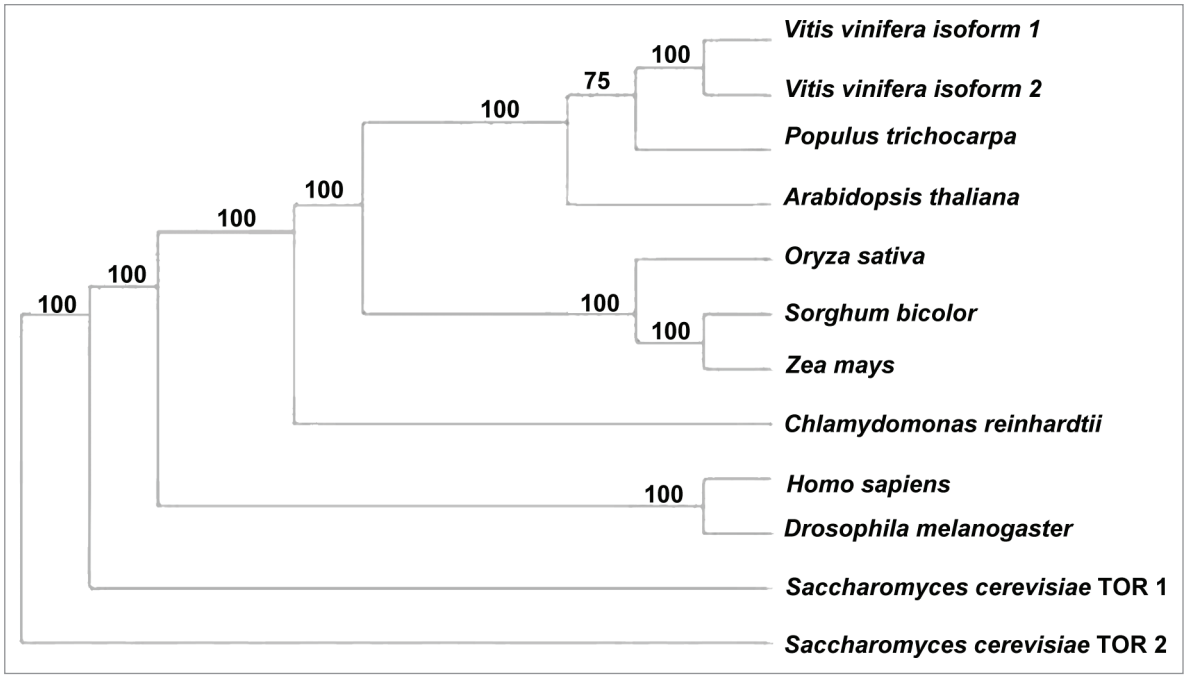

Figure 2. Phylogenetic tree of TOR proteins from different species. Four main groups are formed by the animal kingdom (H.s., D.m.), fungi (S.c.), algae (C.r.) and higher plants. The latter group can be subdivided into grasses (Z.m., S.b., O.s.) and dicotyledons (A.t., V.v., P.t.). The closest homologs of the Arabidopsis TOR are the TOR proteins of *Populus trichocarpa* and the TOR protein isoforms one (identity: 80%, similarity: 88%) and two (identity: 79%, similarity: 87%) of *Vitis vinifera.* In all these plant proteins, the general domain structure as well as the protein sequence is well conserved. The phylogenetic tree was done with the PHYLIP software and is based on a ClustalW multiple alignment of protein sequences. The bootstrap numbers indicate the number of times the group consisting of the protein sequences which are to the right of that fork occurred among the trees, out of 100 trees. All bootstrap values are high, indicating a very robust phylogenetic tree.

TOR Regulatory Network

A number of components of the TOR signaling pathway have been identified, revealing a highly complex network (**Fig. 3**). In mammals, activity of TOR is basically influenced by three major factors, the abundance of insulin-like growth factors, nutrients, as well as the cellular energy status. Changes in these conditions eventually lead to a modification of the tuberous sclerosis complex (TSC) which is a dimer of TSC1 (HAMARTIN) and TSC2 (TUBERIN) and a negative regulator of TOR. Growth factors act through the PI3K pathway and lead to activation of the kinases PDK1 and AKT. Activated AKT inhibits the suppressing action of TSC and thereby activates TOR. The nutrient status of the cell is particularly represented by the abundance of amino acids. The exact mechanism by which nutrient availability is monitored remains to be elucidated. It has been shown, however, that TORC1 is able to sense the cellular energy status through the AMP-activated protein kinase (AMPK).[1]

In plants, alternative signaling pathways must have developed since they seem to lack homologs of TSC1 and TSC2. Yet, some mechanisms appear conserved since homologs of the mammalian PI3K pathway components have been identified in maize. A 20 kDa insulin-related peptide (IGF) was isolated which has been shown to share epitope homology with mammalian insulin. The time of *IGF* expression coincides with the onset of fast growth during germination.[30] When maize seeds were treated with isolated IGF, a significant increment in coleoptile and root lengths could be detected in comparison with the untreated control. Furthermore, IGF and insulin stimulated selectively the translation of ribosomal proteins and led to a selective recruitment of translation apparatus mRNAs into polysomes. These effects could be blocked by treatment with rapamycin, suggesting the involvement of TOR signaling in the response to IGF or insulin.[17]

The translationally controlled tumor protein (TCTP) is an important component of the TOR signaling network. A large number of studies in various organisms have related TCTP to diverse cellular processes such as apoptosis, microtubule organization or ion homeostasis and several interacting proteins (e.g., Polo Kinase, Tubulin and Na^+/K^+-ATPase) were identified. TCTP is a guanine exchange factor of the small GTPase Rheb which has been shown to influence TOR in Drosophila. The Arabidopsis genome also codes for a TCTP. A knockout of *TCTP* leads to a male gametophytic phenotype with normal pollen formation and germination but impaired pollen tube growth, explaining the inability to find homozygous *tctp* mutants. Silencing of *TCTP* by RNA interference slows vegetative growth and leaf expansion is reduced due to a smaller cell size. Lateral root formation is reduced and root hair development is impaired. These lines also show decreased sensitivity to an exogenously applied auxin analog and have elevated levels of endogenous auxin.[31] Auxins are important plant growth stimulating hormones and TCTP seems to represent a link between these general growth promoting factors and TOR signaling.

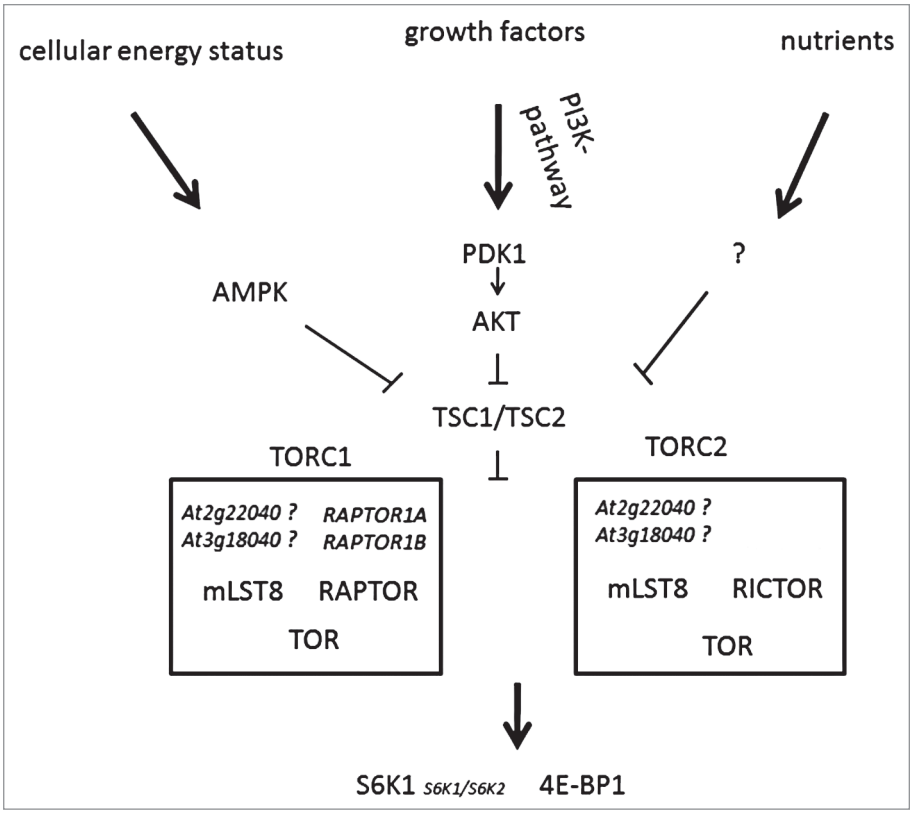

Figure 3. Simplified TOR signaling network with known Arabidopsis homologs in Italic writing. Activity of TOR is influenced by abundance of growth factors and nutrients as well as by the cellular energy level. Growth factors act through the PI3K pathway which finally leads to inactivation of the TOR inhibitory complex TSC1/TSC2. The cellular energy status is mostly represented by the abundance of amino acids which lead to the activation of AMPK kinase and to subsequent inactivation of TSC1/TSC2. How nutrient perception and intracellular signaling works in detail still remains to be elucidated. Gene identifiers represent possible Arabidopsis LST8 homologs.

Downstream Targets of TOR

The best studied downstream targets of TOR are the regulators of protein biosynthesis S6K1 (protein S6 kinase 1) and 4E-BP1 (eukaryotic initiation factor 4E (eIF-4E) binding protein 1). Phosphorylation of S6K1 and 4E-BP1 by TOR leads to a higher rate of protein synthesis.[1] The Arabidopsis genome encodes two S6 kinase homologs, S6K1 and S6K2, and an in vivo assay showed that RAPTOR1B is interacting with S6K1. Furthermore, S6K1 activity is reduced upon application of osmotic stress and Arabidopsis plants overexpressing *S6K1* are overly sensitive to osmotic stress, a process that is strongly influenced by the TOR pathway.[27]

In yeast and mammals, protein phosphatase 2A (PP2A) is a regulator of cell growth in coordination with nutrient availability and environmental conditions. TAP42 is a regulatory subunit of PP2A and a downstream effector of TOR. Recently, it could be shown that the Arabidopsis TAP42 homolog TAP46 has similar functions as TAP42, positively affects cell growth and can be phosphorylated in vitro by TOR. This suggests that PP2A is a downstream target of TOR signaling via TAP46 and provides evidence for a direct interaction of the two proteins.[32]

Effect of the TOR Pathway on Cell Wall Development

Plants are surrounded by a rigid cell wall that must be able to intermittently enlarge to enable cell expansion. Therefore, TOR signaling is likely to be involved in the coordination of this aspect of cell growth. Indeed, work in yeast suggests a role of the TOR pathway in cell wall integrity signaling.[33] Studies on the Arabidopsis cell wall formation mutant *rol5* (*repressor of lrx1_5*) provided first evidence for such a function in plants. The *rol5* mutant was discovered as a suppressor of the cell wall formation mutant *lrx1*. LRX1 (LRR-extensin 1) is involved in cell wall formation and the *lrx1* mutant shows a defect in the formation of root hairs.[34,35] While an *lrx1 rol5* double mutant shows a suppressed *lrx1* phenotype, i.e., wild-type like root hair development, the *rol5* single mutant develops shorter root hairs, shorter root epidermal cells and exhibits altered cell wall structures compared with the wild type.[20] The ROL5 protein shows 54% identity and 70% similarity to Ncs6p/Tuc1p of yeast (*Saccharomyces cerevisiae*), subsequently referred to as Ncs6p. Ncs6p-like proteins of different organism share conserved motifs, including a PP-loop domain with ATP pyrophosphatase activity, which are also conserved in ROL5. A Δ*ncs6* mutant is hypersensitive to rapamycin, which suggests a potential function of Ncs6p in TOR signaling.[36-39] A second phenotype of the Δ*ncs6* mutant is the lack of thiolated uridines in the wobble position of a subset of tRNAs. This modification is not crucial for cell viability but for efficient protein translation.[40] A full-length *ROL5* construct was able to complement for these phenotypes of the Δ*ncs6* mutant, demonstrating that ROL5 is functionally similar to Ncs6p. Interestingly, it has been shown recently that an accumulation of tRNAs in the nucleus leads to reduced TORC1 activity and upregulated autophagy in human fibroblasts.[41] This finding suggests a link between TOR signaling and tRNAs abundance. To test if ROL5 is involved in TOR signaling in Arabidopsis, *rol5* and wild-type plants were rendered rapamycin-sensitive by overexpression of the yeast *ScFKBP12*. In Arabidopsis, rapamycin-sensitivity causes reduction in root growth.[19] At a low concentration of rapamycin that does not cause a reduction in root growth in the wild type expressing *ScFKBP12* (1.7 ± 0.01 vs. 1.6 ± 0.03 cm without and with rapamycin, respectively; $p = 0.05$), *rol5* mutants with the very same *ScFKBP12* transgene insertion showed significantly shorter roots (1.1 ± 0.02 vs. 0.8 ± 0.01 cm without and with rapamycin, respectively; $p = 0.05$). This demonstrates that the *rol5* mutation renders plants hypersensitive to

rapamycin and suggests a role of ROL5 in TOR signaling. When wild-type plants expressing *ScFKBP12* were treated with rapamycin, they developed alterations in cell wall structures comparable to those of the *rol5* single mutant.[20] Finally, the *lrx1* root hair phenotype could be suppressed through interfering with TOR signaling by rapamycin. (**Fig. 4**).[20] Together, these findings suggest that TOR signaling has an influence on cell wall formation and that ROL5 is a component in this aspect of the TOR pathway in Arabidopsis.

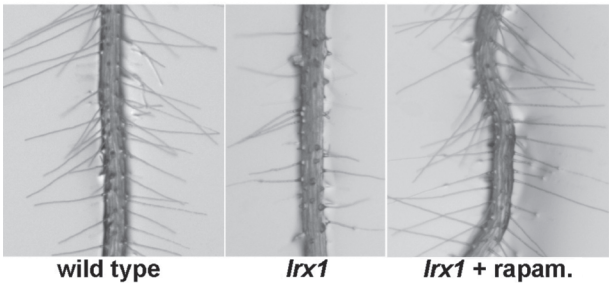

Figure 4. Comparison of Arabidopsis wild-type and *lrx1* mutant plants illustrate the severe root hair formation phenotype in *lrx1*. If *lrx1* plants overexpressing yeast *FKBP12* are treated with rapamycin, the *lrx1* phenotype is suppressed and wild-type like root hairs develop.

Future Perspectives

In animals and yeast, the TOR pathway received tremendous attention due to its importance in regulating cell growth and, consequently, as a potential target for medical applications. In plants, we are only beginning to understand the molecular mechanisms of TOR signaling. While certain proteins are obviously conserved between yeast, animals, and plants, a number of components seem not encoded in the plant genomes analyzed so far. It will be highly interesting to understand the alternative methods developed in plants to relay signals in the TOR pathway. Some of these plant-specific proteins might have functions that are particular to plants such as the development of the cell wall. The molecular-genetic tools available in plants provide an excellent starting point for genetically identifying new components of the TOR pathway that might become valuable means to modify TOR signaling in mammalian cells.

Acknowledgments

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References

1. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell 2006; 124:471-84; PMID:16469695; DOI: 10.1016/j.cell.2006.01.016.
2. Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature 2007; 450:736-40; PMID:18046414; DOI:10.1038/nature06322.
3. Gapper C, Dolan L. Control of plant development by reactive oxygen species. Plant Physiol 2006; 141:341-5; PMID:16760485; DOI:10.1104/pp.106.079079.
4. Schmelzle T, Hall MN TOR, a central controller of cell growth. Cell 2000; 103:253-62; PMID:11057898; DOI:10.1016/S0092-8674(00)00117-3.
5. Huang S, Bjornsti MA, Houghton PJ. Rapamycins—mechanism of action and cellular resistance. Cancer Biol Ther 2003; 2:222-32; PMID:12878853; DOI:10.4161/cbt.2.3.360.
6. Schieke SM, Finkel T. Mitochondrial signaling, TOR and life span. Biol Chem 2006; 387:1357-61; PMID:17081107; DOI:10.1515/BC.2006.170.
7. Chong ZZ, Shang YC, Zhang LJ, Wang SH, Maiese K. Mammalian target of rapamycin Hitting the bull's-eye for neurological disorders. Oxid Med Cell Longev 2010; 3:374-91; PMID:21307646; DOI:10.4161/oxim.3.6.14787.
8. Gabardi S, Baroletti SA. Everolimus: A proliferation signal inhibitor with clinical applications in organ transplantation. Pharmacotherapy 2010; 30:1044-56; PMID:20874042; DOI:10.1592/phco.30.10.1044.
9. McMahon G, Weir MR, Li XC, Mandelbrot DA. The evolving role of mTOR inhibition in transplantation tolerance. J Am Soc Nephrol 2011; 22:408-15; PMID:21355051; DOI:10.1681/ASN.2010040351.
10. Knutson BA. Insights into the domain and repeat architecture of target of rapamycin. J Struct Biol 2010; 170:354-63; PMID:20060908; DOI:10.1016/j.jsb.2010.01.002.
11. Soulard A, Cohen A, Hall MN. TOR signaling in invertebrates. Curr Opin Cell Biol 2009; 21:825-36; PMID:19767189; DOI:10.1016/j.ceb.2009.08.007.
12. Dames SA, Mulet JM, Rathgeb-Szabo K, Hall MN, Grzesiek S. The solution structure of the FATC domain of the protein kinase target of rapamycin suggests a role for redox-dependent structural and cellular stability. J Biol Chem 2005; 280:20558-64; PMID:15772072; DOI:10.1074/jbc.M501116200.
13. Ren M, Qiu SQ, Venglat P, Xiang DQ, Feng L, Selvaraj G, et al. Target of Rapamycin regulates development and ribosomal RNA expression through kinase domain in Arabidopsis. Plant Physiol 2011; 155:1367-82; PMID:21266656; DOI:10.1104/pp.110.169045.
14. Menand B, Desnos T, Nussaume L, Berger F, Bouchez D, Meyer C, et al. Expression and disruption of the Arabidopsis TOR (target of rapamycin) gene. Proc Natl Acad Sci USA 2002; 99:6422-7; PMID:11983923; DOI:10.1073/pnas.092141899.
15. Agredano-Moreno LT, Reyes de la Cruz H, Patricio Martinez-Castilla L, Sanchez de Jimenez E. Distinctive expression and functional regulation of the maize (*Zea mays* L.) *TOR* kinase ortholog. Mol Biosyst 2007; 3:794-802; PMID:17940662; DOI:10.1039/b705803a.
16. Crespo JL, Diaz-Troya S, Florencio FJ. Inhibition of target of rapamycin signaling by rapamycin in the unicellular green alga *Chlamydomonas reinhardtii*. Plant Physiol 2005; 139:1736-49; PMID:16299168; DOI:10.1104/pp.105.070847.
17. Sotelo R, Garrocho-Villegas V, Aguilar R, Calderon ME, de Jimenez ES. Coordination of cell growth and cell division in maize (*Zea mays* L.) relevance of the conserved TOR signal transduction pathway. In Vitro Cell Dev Biol Plant 2010; 46:578-86; DOI:10.1007/s11627-010-9293-8.
18. Xu Q, Liang S, Kudla J, Luan S. Molecular characterization of a plant FKBP12 that does not mediate action of FK506 and rapamycin. Plant J 1998; 15:511-9; PMID:9753776; DOI:10.1046/j.1365-313X.1998.00232.x.
19. Sormani R, Yao L, Menand B, Ennar N, Lecampion C, Meyer C, et al. *Saccharomyces cerevisiae* FKBP12 binds *Arabidopsis thaliana* TOR and its expression in plants leads to rapamycin susceptibility. BMC Plant Biol 2007; 7:26; PMID:17543119; DOI:10.1186/1471-2229-7-26.
20. Leiber RM, John F, Verhertbruggen Y, Diet A, Knox JP, Ringli C. The TOR pathway modulates the structure of cell walls in arabidopsis. Plant Cell 2010; 22:1898-908; PMID:20530756; DOI:10.1105/tpc.109.073007.
21. Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS, et al. A mammalian protein targeted by G_1-arresting rapamycin-receptor complex. Nature 1994; 369:756-8; PMID:8008069; DOI:10.1038/369756a0.
22. Chiu MI, Katz H, Berlin V. RAPT1, a mammalian homolog of yeast TOR, interacts with the FKBP12 rapamycin complex. Proc Natl Acad Sci USA 1994; 91:12574-8; PMID:7809080; DOI:10.1073/pnas.91.26.12574.
23. Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W. Genevestigator. Arabidopsis microarray database and analysis toolbox. Plant Physiol 2004; 136:2621-32; PMID:15375207; DOI:10.1104/pp.104.046367.
24. Deprost D, Yao L, Sormani R, Moreau M, Leterreux G, Nicolai M, et al. The Arabidopsis TOR kinase links plant growth, yield, stress resistance and mRNA translation. EMBO Rep 2007; 8:864-70; PMID:17721444; DOI:10.1038/sj.embor.7401043.
25. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol 2011; 13:132-41; PMID:21258367; DOI:10.1038/ncb2152.
26. Liu Y, Bassham DC. TOR Is a Negative regulator of autophagy in *Arabidopsis thaliana*. PLoS ONE 2010; 5:11883; PMID:20686696.
27. Mahfouz MM, Kim S, Delauney AJ, Verma DPS. Arabidopsis Target of Rapamycin interacts with Raptor, which regulates the activity of S6 kinase in response to osmotic stress signals. Plant Cell 2006; 18:477-90; PMID:16377759; DOI:10.1105/tpc.105.035931.
28. Deprost D, Truong HN, Robaglia C, Meyer C. An Arabidopsis homolog of RAPTOR/KOG1 is essential for early embryo development. Biochem Biophys Res Commun 2005; 326:844-50; PMID:15607746; DOI:10.1016/j.bbrc.2004.11.117.
29. Anderson GH, Veit B, Hanson MR. The Arabidopsis *AtRaptor* genes are essential for post-embryonic plant growth. BMC Biol 2005; 3:12; PMID:15845148; DOI:10.1186/1741-7007-3-12.

30. Dinkova TD, de la Cruz HR, Garcia-Flores C, Aguilar R, Jimenez-Garcia LF, de Jimenez ES. Dissecting the TOR-S6K signal transduction pathway in maize seedlings: relevance on cell growth regulation. Physiol Plant 2007; 130:1-10; DOI:10.1111/j.1399-3054.2007.00862.x.
31. Berkowitz O, Jost R, Pollmann S, Maslea J. Characterization of TCTP, the Translationally Controlled Tumor Protein, from *Arabidopsis thaliana.* Plant Cell 2008; 20:3430-47; PMID:19060111; DOI:10.1105/tpc.108.061010.
32. Ahn CS, Han JA, Lee HS, Lee S, Pai HS. The PP2A regulatory subunit Tap46, a component of the TOR signaling pathway, modulates growth and metabolism in plants. Plant Cell 2011; 23:185-209; PMID:21216945; DOI:10.1105/tpc.110.074005.
33. Levin DE. Cell wall integrity signaling in *Saccharomyces cerevisiae.* Microbiol Mol Biol Rev 2005; 69:262-91; PMID:15944456; DOI:10.1128/MMBR.69.2.262-91.2005.
34. Baumberger N, Ringli C, Keller B. The chimeric leucine-rich repeat/extensin cell wall protein LRX1 is required for root hair morphogenesis in *Arabidopsis thaliana.* Genes Dev 2001; 15:1128-39; PMID:11331608; DOI:10.1101/gad.200201.
35. Baumberger N, Steiner M, Ryser U, Keller B, Ringli C. Synergistic interaction of the two paralogous Arabidopsis genes *LRX1* and *LRX2* in cell wall formation during root hair development. Plant J 2003; 35:71-81; PMID:12834403; DOI:10.1046/j.1365-313X.2003.01784.x.
36. Bork P, Koonin EV. A P-Loop-like motif in a widespread ATP pyrophosphatase domain—implications for the evolution of sequence motifs and enzyme activity. Proteins 1994; 20:347-55; PMID:7731953; DOI:10.1002/prot.340200407.
37. Björk GR, Huang B, Persson OP, Bystrom AS. A conserved modified wobble nucleoside (mcm(5)s(2)U) in lysyl-tRNA is required for viability in yeast. RNA 2007; 13:1245-55; PMID:17592039; DOI:10.1261/rna.558707.
38. Chan TF, Carvalho J, Riles L, Zheng XFS. A chemical genomics approach toward understanding the global functions of the target of rapamycin protein (TOR). Proc Natl Acad Sci USA 2000; 97:13227-32; PMID:11078525; DOI:10.1073/pnas.240444197.
39. Goehring AS, Rivers DM, Sprague GF. Urmylation: A ubiquitin-like pathway that functions during invasive growth and budding in yeast. Mol Biol Cell 2003; 14:4329-41; PMID:14551258; DOI:10.1091/mbc.E03-02-0079.
40. Leidel S, Pedrioli PGA, Bucher T, Brost R, Costanzo M, Schmidt A, et al. Ubiquitin-related modifier Urm1 acts as a sulphur carrier in thiolation of eukaryotic transfer RNA. Nature 2009; 458:228-32; PMID:19145231; DOI:10.1038/nature07643.
41. Huynh LN, Thangavel M, Chen T, Cottrell R, Mitchell JM, Praetorius-Ibba M. Linking tRNA localization with activation of nutritional stress responses. Cell Cycle 2010; 9:3112-8; PMID:20714220; DOI:10.4161/cc.9.15.12525.

2.4 tRNAs and their biological function

The process of protein biosynthesis requires transfer RNA molecules as interpreters of the genetic code. In eukaryotes, twenty aminoacyl-tRNA synthetase genes (*aaRS*), one for every amino acid, are known. These aaRS enzymes catalyse the attachment of the particular amino acid to the 3'-end of the tRNAs for subsequent transfer to the nascent protein chain (Banerjee et al., 2010). Unloaded tRNAs also have functions beyond protein biosynthesis. If the level of available amino acids is low, tRNAs act as regulators of global gene expression. This has been demonstrated in gram-positive bacteria. When sufficient amino acids are available, tRNAs act as anti-terminators on the 5'-leader sequence of mRNAs. This leads to continuing protein translation (Guttierez-Preciado et al., 2009). If amino acid levels are low, unloaded tRNAs are often cleaved, which in consequence leads to reduced protein biosynthesis (Jöchl et al., 2008; Haiser et al., 2008; Hao et al., 2005; 2010). Intriguingly, cleavage of tRNAs is also induced upon oxidative stress in many eukaryotes, including plants. How this cleavage relates to stress signaling still remains to be elucidated (Thompson et al., 2008; 2009). Moreover, there is evidence that nuclear localization or export of tRNAs represents a sensing mechanism for the cellular nutrient status in fibroblasts. It has been shown that retention of tRNAs in the nucleus triggers a starvation-like response. However, it is not clear yet how this signaling works in detail but modification or cleavage of tRNAs by nucleus-specific enzymes represents a feasible mechanism (Huynh et al., 2010). Recently, evidence accumulated that tRNAs influence TOR via a autoregulatory feedback loop. In this model, the amount of available cytoplasmic tRNAs contributes to protect against nutrient limitation by inhibiting TOR (Huynh et al., 2010). This means that a high nutrient availability would lead to stimulation of TOR, promoting the production of tRNAs, which in turn provides a positive feedback, leading to further stimulation of TOR.

2.4.1 Process of tRNA thiolation

It has been demonstrated that tRNAs of the acceptor types $tRNA^{Glu}$, $tRNA^{Lys}$ and $tRNA^{Gln}$ contain 2-thiouridine derivates (s2U) in the wobble position. Species of the acceptor type $tRNA^{Gln}$ and $tRNA^{Lys}$ are modified by 5-methylaminomethyl-2-thiouridine (mcm5s2U) (Rogers et al., 1995**).** The thiolation of tRNAs has been proposed to be important for translational efficiency or the accuracy of codon reading (Rogers et al., 1995). But in the meantime, it has been shown that the modification of tRNAs is also part of the cellular stress and nutritional sensing machinery (Leidel et al. 2009). Right now, six proteins are thought to be needed for thiolation of tRNAs in yeast. These are namely Urm1p, Uba4p, Ncs6p, and Ncs2p with Nfs1p and Tum1p acting upstream as sulphur donors. Uba4p acts as sulphur acceptor from Tum1p and Nfs1p before forming a diacyl bond with Urm1p. Uba4p then releases a thiocarboxylated version of Urm1p. The PP-loop ATPases Ncs6p and Ncs2p are essential for in vivo 2-thiolation modification of mcm5U. But the exact way of 2-thiouridine formation still remains to be elucidated (Leidel et al., 2009; Noma et al., 2009; Wang et al., 2011; Van der Veen et al., 2011). The abundance of 2-thiouridine-modified tRNAs can be visualized on a polyacrylamide gel supplemented with [(N-acryloylamino)-phenyl]mercuric chloride (APM). Thereby it could be demonstrated that yeast *Δuba4*, *Δurm1*, *Δncs2*, or *Δncs6* lack the ability to form 2-thiouridine modifications (Leidel et al., 2009). These mutations have been shown to be non-lethal, but yeast cells are affected in invasive growth and in budding, which is also reflected by the fact that double mutants with *Δcla4* are lethal. Cla4p is a p21-activated kinase which acts during budding. In addition, *Δuba4*, *Δurm1*, *Δncs2*, or *Δncs6* render cells hypersensitive to the drug rapamycin, which is a potent inhibitor of TOR signaling (Goehring et al., 2003a; Schlieker et al., 2008; Leidel et al., 2009). TOR is a central relay for nutrient-dependent growth activation. Since it could be shown that the localization of tRNAs is linked to nutritional stress responses, this gives further evidence that tRNAs are involved in TOR-dependent nutrient sensing (Wullschleger et al., 2006; Leidel et al., 2009; Huynh et al., 2010).

2.4.2 Protein urmylation

Ubiquitin-related modifier 1 (Urm1p) is a member of ubiquitin like proteins (UBLs). A hallmark of UBLs is a conserved core ß-grasp-fold structural motif and a conserved C-terminal diglycine (Furukawa et al., 2000; Wang et al., 2011). As shown in Figure 2, Urm1p is involved in tRNA thiolation.

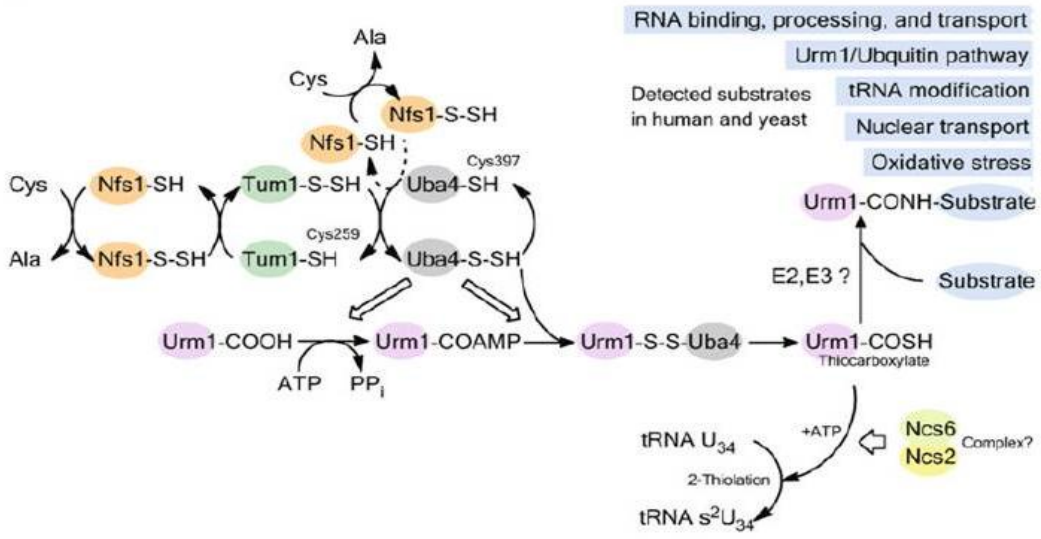

Figure 2 Pathway of 2-thiouridine modifications. Uba4p is sulfur modified by Nfs1p, Tum1p and subsequently thiocarboxylates Urm1p. This stage is a crossing point of the pathway since thiocarboxylated Urm1p can be used for protein modification or for transferring the thio-group to tRNAs (figure from Wang et al. 2011).

In addition, evidence for Urm1p having a function in protein modification (urmylation) is accumulating. Intriguingly, *Δurm* mutants are hypersensitive to rapamycin. This suggests also a link to TOR signaling (Leidel et al., 2009). In yeast, conjugation of Urm1p to target proteins needs the action of activating enzyme E1 (Uba4p in *S. cerevisiae*) (Goehring et al., 2003b).

Covalent attachment of ubiquitin or UBLs to proteins regulates a lot of different cellular processes. In yeast UBL Urm1p has been shown to play a role in budding, nutrient sensing, high temperature sensitivity or antioxidant stress response. The latter is also reflected by attachment of Urm1p to the oxidative stress-related protein Ahp1p, and posttranslational modification of the elongator subunit (Furukawa et al. 2000; Fichtner et al., 2003; Goehring et al., 2003a; 2003b ; Rubio-Texeira, 2007). In addition, it has been shown recently in human cells that Urm1p is conjugated to 21 target proteins upon oxidative stress (Van der Veen et al., 2011). Oxidative stress represents a severe threat to the integrity of the cell. Cells need sensing mechanisms which are able to sense a thread and to trigger a defense response (Fuchs et al., 2009). TOR signaling, as well as the conjugation of Urm1p to target proteins have been shown to be influenced by oxidative stress. Furthermore, the Urm1p-pathway mutants *Δurm*, *Δuba4*, *Δncs6* and *Δncs2* are hypersensitive to rapamycin (Goehring et al., 2003b). Therefore, an involvement of protein urmylation in TOR signaling and oxidative stress response is indicated. Based on this, it can be speculated whether the Urm1p-pathway represents a mechanism for relaying oxidative stress signals to TOR. Consequently, TOR signaling needs to be linked to the CWI pathway in order to sustain cell wall integrity upon detection of stress. In *S. cerevisiae*, this was in fact demonstrated by the finding that the *Δtor2* mutant growth arrest and actin depolarization defects could be suppressed by overexpression of CWI components (Levin, 2005). In addition, evidence accumulated that the cross-talk between TOR and CWI is most likely to be established by TOR dependent regulation of small GTPases which in turn regulate the MAPK cascade (Fuchs et al., 2009). This pathway is well known to induce changes in the transcription level of cell wall biosynthesis genes. In yeast, small GTPase Rho1 also directly activates the glucan synthase Fks1 which facilitates the production of 1,3-β-D-glucan, a major cell wall component in yeast (Fuchs et al., 2009). The idea that small GTPases represent the link between TOR and CWI was corroborated in *C. albicans*. When Δ*rhb1* mutants, affected in a small GTP binding protein, were treated with cell wall disrupting agents or the TOR inhibiting drug rapamycin, a hypersensitivity phenotype was observed

(Tsao et al., 2009). This makes small GTPases and especially members of the Rho family very promising candidates for linking TOR signaling and CWI sensing also in plants.

2.5 Rho family proteins

The Rho family of GTP binding proteins is composed of three subfamilies (Cdc42, Rac, Rho) and further, phyla-specific subfamilies. Cdc42 and Rho are present in yeast and most animals whereas Rac is animal-specific (Yang, 2002). The members of the Rho family are known to be signaling molecules in yeast and in mammals. The first plant Rho-related proteins were identified in pea (Young and Watson, 1993). Subsequently, members of this family in plants were named Rho of plants (ROPs). Like all Rho family members, ROPs are small GTPases which exist in two functional states, a GTP-bound active state, and a GDP-bound inactive state. In *Arabidopsis thaliana*, 11 ROP family members have been identified. By phylogenetic analysis, ROPs were classified into four groups. ROP 1-6 belong to group IV, ROP 7 to group III, ROP 9-11 to group II, and ROP 8 to group I (Yang, 2002).

2.5.1 Function of ROPs

rop mutants have been a major tool for assigning functions to specific ROPs in Arabidopsis, cotton, rice, and maize. For this work, I like to focus on Arabidopsis ROPs of group IV. Members of this group mainly control growth-related processes like actin dynamics, root hair development, and ABA responses (Yang, 2002; Klahre et al., 2006; Klahre and Kost, 2006; Kost, 2008). Cell growth requires well-organized microtubuli which promote cell elongation and restrict radial expansion. Analysis of the Arabidopsis *rop6-1* mutant revealed a loss in microtubule organization. Overexpression of *ROP6* in turn enhances microtubule organization. This subsequently leads to a loss of the jigsaw-puzzle appearance of pavement cells (Fu et al., 2009). Recently, an auxin-controlled pathway of ROP activation was postulated. It could be shown that auxin is controlling the leaf interdigitating growth of Arabidopsis epidermal cells by coordinated activation of ROP2 and ROP6 (Xu et al., 2010).

2.5.2 ROP mediated polar cell growth

Spatially restricted signaling of ROPs at certain domains of the plasma membrane has been shown to be essential for polarized cell growth (Kost, 2008). The role of ROP-regulating proteins in this process is not very well understood, yet. Currently, three conserved classes of proteins are known to be regulators of ROP activity in yeast, humans, and plants. These are GEFs (guanine nucleotide exchange factors), GAPs (GTPase activating proteins), and GDIs (guanine nucleotide dissociation inhibitors). The latter mediate the release of GDP, and stabilize the binding pocket for subsequent binding of GTP which occurs at higher intracellular levels than GDP (Thomas et al., 2009). GAP proteins are speeding up the intrinsic GTP hydrolysis process of ROPs, thereby inactivating them. GDIs are also thought to be negative regulators of ROP activity. They are removing the ROP proteins from the membrane and form heterodimers with ROPs, thereby retaining them in the cytoplasm (Manneville et al., 2002; Kost, 2008). On the basis of this, a model for ROP-regulated polar cell growth was proposed. GTP-bound ROP GTPases accumulate at the apical membrane of polarly growing cells. Due to the apical localization of activating GEFs, and the localization of inactivating GAPs at the flanks, ROPs are only active at the apex (Klahre and Kost, 2006). GDIs in turn mediate the shuttling from sites of activation to sites of inactivation (Kost, 2008). This tip-localized ROP activity then mediates spatially restricted growth by regulating cytoskeletal organization and membrane trafficking. In Arabidopsis, it could be shown that indeed PRONEs (Arabidopsis GEFs) colocalize with active ROPs at the apex of pollen tubes (Gu et al., 2006). They have also been shown to influence pollen tube as well as root hair growth, root development, and the response to abiotic stress (Gu et al., 2006; Shin et al., 2010; Mucha et al., 2011; Riely et al., 2011). In tobacco pollen tubes, overexpression of GDI transferred the ROP GTPase NtRAC5 to the cytoplasm, and inhibited pollen tube growth (Klahre et al., 2006). In summary, ROP signaling represents an important multi-level regulatory system. Like Arabidopsis ROPs, the yeast Rho family member Rho1 is involved in the organization of the actin cytoskeleton and site directed

growth. Furthermore, Rho1 is also involved in CWI sensing and has been shown to be influenced by TOR (Fuchs et al., 2009). In Arabidopsis, FERONIA has been linked to CWI and was suggested to be an upstream regulator of RAC/ROP signaling (Duan et al., 2010; Hamann, 2011). Therefore, Arabidopsis ROPs are likely to be involved in CWI maintenance and are promising candidates for linking CWI and TOR.

3 Aim of the study

The *lrx1* mutation induces a severe root hair phenotype in Arabidopsis (Baumberger et al., 2001). A suppressor screen on *lrx1* was performed resulting in the isolation of several repressors of *lrx1* (*rol* mutants), including *rol5*. In yeast, the ROL5 homolog Ncs6p is linked to TOR signaling and tRNA thiolation (Goehring et al., 2003). This raised the question whether ROL5 is performing similar functions in Arabidopsis. Furthermore, we wanted to understand how the *rol5* mutation is able to suppress *lrx1*.

Following the finding that *rol5* most likely suppresses *lrx1* via a TOR-mediated modification of cell wall structures, we aimed at understanding the molecular network ROL5 is involved in and the connection to the TOR pathway.

4 Results

4.1 The TOR pathway modulates the structure of cell walls in Arabidopsis

Experiments performed by Florian John
Measurement of root lengths and trichoblast lengths.
Analysis of tRNA thiolation in yeast cells and Arabidopsis.
Complementation of the *Δncs6* rapamycin growth phenotype with *ROL5* and *rol5-1*.
Suppression of the *lrx1* phenotype by rapamycin.
Immunolabeling of cell walls.
Treatment of *rol5-1* with ROS and ROS scavenger.
ROS staining of *rol5-1*.

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The TOR Pathway Modulates the Structure of Cell Walls in *Arabidopsis* [W]

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Plant cell growth is limited by the extension of cell walls, which requires both the synthesis and rearrangement of cell wall components in a controlled fashion. The target of rapamycin (TOR) pathway is a major regulator of cell growth in eukaryotes, and inhibition of this pathway by rapamycin reduces cell growth. Here, we show that in plants, the TOR pathway affects cell wall structures. LRR-extensin1 (LRX1) of *Arabidopsis thaliana* is an extracellular protein involved in cell wall formation in root hairs, and *lrx1* mutants develop aberrant root hairs. *rol5* (for *repressor of lrx1*) was identified as a suppressor of *lrx1*. The functionally similar ROL5 homolog in yeast, Ncs6p (needs Cla4 to survive 6), was previously found to affect TOR signaling. Inhibition of TOR signaling by rapamycin led to suppression of the *lrx1* mutant phenotype and caused specific changes to galactan/rhamnogalacturonan-I and arabinogalactan protein components of cell walls that were similar to those observed in the *rol5* mutant. The ROL5 protein accumulates in mitochondria, a target of the TOR pathway and major source of reactive oxygen species (ROS), and *rol5* mutants show an altered response to ROS. This suggests that ROL5 might function as a mitochondrial component of the TOR pathway that influences the plant's response to ROS.

INTRODUCTION

Plant cell growth is tightly linked to the expansion of the cell wall. Cell walls are complex structures that resist internal turgor pressure and, for cell enlargement to take place, have to incorporate new material and rearrange internal linkages between the different components (Martin et al., 2001). In dicotyledonous plants, the primary cell wall is composed of cellulose microfibrils that are interconnected by hemicelluloses, mainly xyloglucan. This is considered to be the load-bearing structure and is embedded in a matrix of pectic polysaccharides (Carpita and Gibeaut, 1993). The pectic matrix has three major components: homogalacturonan, rhamnogalacturonan-I (RGI), which contains side chains of galactan and arabinan, and rhamnogalacturonan-II. Pectins influence cell wall rigidity and strength as well as cell–cell adhesion. In addition, RGI regulates wall porosity, which in turn influences the mobility of cell wall–modifying proteins and, thus, cell wall expansion (Baron-Epel et al., 1988; Ridley et al., 2001; Willats et al., 2001; McCartney et al., 2003). Structural cell wall proteins such as hydroxyproline-rich glycoproteins (HRGPs) influence the mechanical properties of cell walls but can also be involved in cell elongation and signaling as exemplified by arabinogalactan proteins (AGPs). These are GPI-anchored proteins of the HRGP family that are extensively glycosylated with arabinose and galactose (Ding and Zhu, 1997; Majewska-Sawka and Nothnagel, 2000; van Hengel and Roberts, 2002).

The structure of cell walls, which influences the cell walls' properties, is in a constant flow of remodeling as it adapts to the prevailing functional requirements. Therefore, plants have evolved a sensing system to monitor cell wall composition and to regulate cell wall modification and restructuring. These activities are likely to involve transmembrane or membrane-anchored proteins. Receptor-like kinases, such as THESEUS and wall-associated kinases, have been shown to sense and modify cell elongation (Kohorn et al., 2006; Hematy et al., 2007), as have lectins and GPI-anchored proteins, such as AGPs (Humphrey et al., 2007; Hematy and Höfte, 2008). LRR-extensins (LRXs) are extracellular proteins consisting of an N-terminal leucine-rich repeat domain and a C-terminal extensin domain typical of HRGPs (Baumberger et al., 2003a). This particular structure suggests that LRX proteins might have a signaling or regulatory function during cell wall development (Ringli, 2005). Indeed, *Arabidopsis thaliana LRX1* is predominantly expressed in root hairs, and *lrx1* mutants develop defective cell walls resulting in aberrant root hair formation (Baumberger et al., 2001, 2003b).

The TOR (for target of rapamycin) pathway is a major growth regulator in eukaryotes that senses nutrient availability and growth stimulators, regulates the translational machinery, and modulates cell growth. The Ser/Thr kinase TOR is central to the TOR pathway and is inhibited by the specific inhibitor rapamycin,

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resulting in reduced cell growth. Rapamycin inhibits the TOR kinase by forming a ternary complex with the immunophilin protein FKBP12 (FK506 binding protein 12) and TOR (Huang et al., 2003; Wullschleger et al., 2006). An important function of the TOR pathway is the regulation of mitochondrial activity and, hence, the production of reactive oxygen species (ROS), which affect life span (Schieke and Finkel, 2006; Cunningham et al., 2007) and, in plants, have an impact on oxidative stress, cell wall extension, and cell growth (for review, see Gapper and Dolan, 2006; Rhoads et al., 2006).

Recent analyses in *Candida albicans* have provided evidence for the participation of the TOR pathway in cell wall integrity sensing in yeast (Tsao et al., 2009). Numerous components of the TOR pathway were identified in yeast based on rapamycin hypersensitivity of the corresponding mutants (Chan et al., 2000). Mutations in *NCS6* (*needs Cla4 to survive 6*) of yeast induce rapamycin hypersensitivity and influence cell growth under nutrient-limited conditions (Chan et al., 2000; Goehring et al., 2003a). Recently, Ncs6p has been shown to be important for the modification of cytoplasmic tRNAs. tRNAs are frequently modified, mostly at the wobble position (position 34) or next to and 3′ of the anticodon (position 37). tRNAs specific for Glu, Glc, and Lys have a 2-thiouridine derivative as wobble nucleoside, which helps to effectively read the corresponding codons on the mRNAs (Björk et al., 2007). Ncs6p and homologous proteins in other organisms are involved in the thiolation of U34, and mutations in the corresponding genes lead to the absence of thiolation (Björk et al., 2007; Schlieker et al., 2008; Leidel et al., 2009). Even though mutating *ncs6* only affects cytoplasmic tRNAs (Noma et al., 2009), Ncs6p is also found in mitochondria (Huh et al., 2003). Dual localization of proteins in different compartments frequently has been observed (Krause and Krupinska, 2009). Thus, it remains to be shown whether the effect of the *ncs6* mutant on TOR signaling is an indirect effect induced by the lack of tRNA modification or a second activity of the protein, reflected by its presence in mitochondria.

The TOR pathway has also been identified in plants, and some of the proteins involved in this process have been characterized (Anderson et al., 2005; Deprost et al., 2005; Ingram and Waites, 2006; Mahfouz et al., 2006). While a *tor* knockout mutant in *Arabidopsis* is embryo-lethal, modified *TOR* expression strongly influences plant growth, emphasizing the importance of TOR during plant development (Menand et al., 2002; Deprost et al., 2007). *Arabidopsis* is not sensitive to the specific TOR inhibitor rapamycin as rapamycin cannot form the ternary complex with FKBP12 and TOR. However, expression of yeast *FKBP12* induces rapamycin sensitivity in *Arabidopsis* (Mahfouz et al., 2006; Sormani et al., 2007).

Here, we provide evidence for a role of the plant TOR pathway in modulating cell wall structures. A suppressor screen on the root hair cell wall formation mutant *lrx1* resulted in the identification of the *rol5* (for *repressor of lrx1*) mutant. The *rol5* mutation induced changes in cell wall structure that might be the basis of suppression of *lrx1*. ROL5 is functionally similar to Ncs6p, which influences TOR signaling in yeast and is required for the modification of tRNAs in *Arabidopsis*. Interfering with TOR signaling by the addition of rapamycin in yeast *FKBP12*-expressing *lrx1* mutant plants relieved the *lrx1* root hair phenotype and induced specific changes in cell wall structure similar to *rol5*. Together, these data indicate that interfering with TOR signaling induces changes in cell walls and provide evidence for a role of the TOR pathway in the regulation of cell wall structure and properties.

RESULTS

Identification of *rol5*, a Suppressor of the *lrx1* Root Hair Phenotype

As a result of the defective cell wall structure, *lrx1* mutants form root hairs that are short and deformed and frequently burst (Figures 1A and 1B). To identify new loci that are involved in regulating cell wall formation and structure, a suppressor screen was performed on the *lrx1* mutant. As described previously (Diet et al., 2006), an *lrx1* missense allele was used for ethyl methanesulfonate (EMS) mutagenesis, and M2 seedlings displaying a suppressed *lrx1* phenotype were isolated. The *rol5-1* mutant was identified in this screen as it suppressed the *lrx1* phenotype. *lrx1 rol5-1* double mutants developed root hairs that were comparable to those of wild-type seedlings (Figure 1C). The *rol5-1* mutation was found to be recessive, since the F1 generation of a backcross with *lrx1* developed an *lrx1* phenotype and seedlings

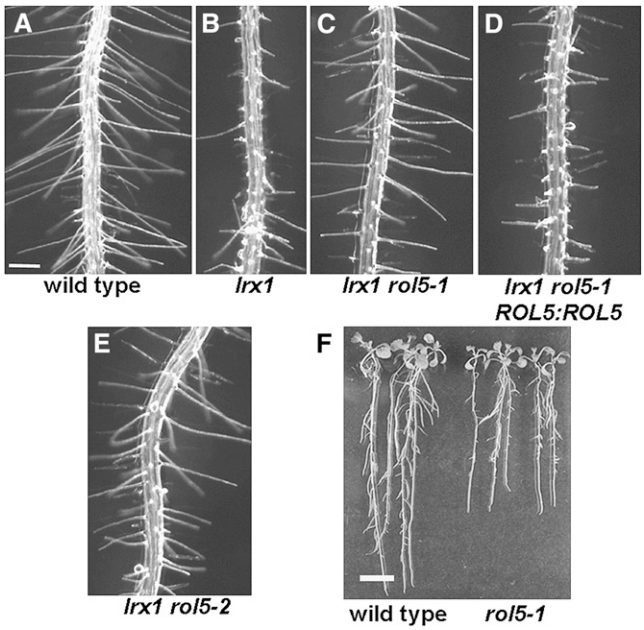

Figure 1. Suppression of the *lrx1* Root Hair Phenotype by Mutations in *rol5*.

Seedlings were grown for 5 d (**[A]** to **[E]**) and 10 d **(F)** in a vertical orientation. The wild type **(A)** developed regular root hairs, whereas root hairs of the *lrx1* mutant **(B)** were severely deformed. The EMS missense allele *rol5-1* **(C)** was complemented with a *ROL5* genomic clone, inducing an *lrx1*-like phenotype **(D)**. The *rol5-2* T-DNA knockout mutant **(E)** also suppressed the *lrx1* mutant phenotype. The *rol5* mutation **(F)** leads to shorter roots as shown for *rol5-1*. Bars = 0.5 mm in **(A)** to **(E)** and 10 mm in **(F)**.

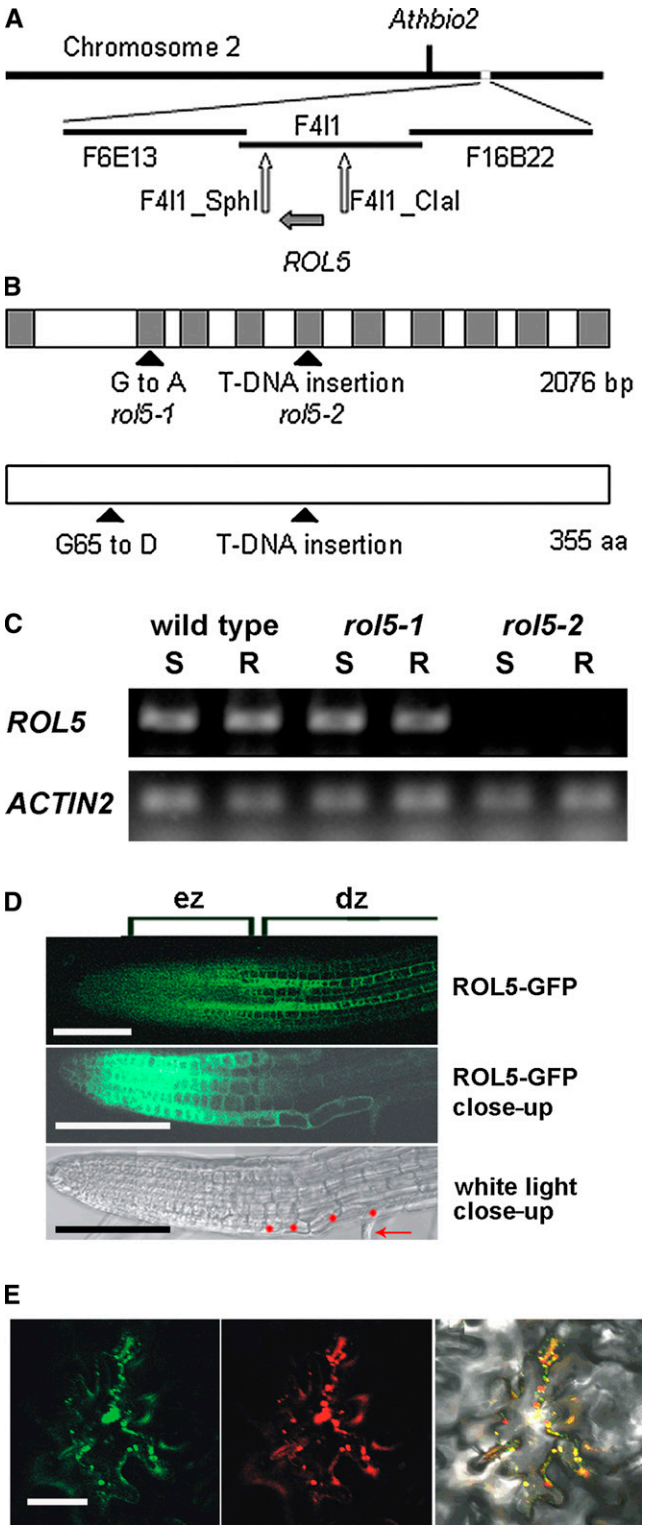

Figure 2. Identification of the *ROL5* Locus.

(A) The *rol5* locus was identified by map-based cloning on the long arm of chromosome 2, south of *Athbio2*. BAC clones in the region of *ROL5* are indicated. For mapping, cleaved-amplified polymorphic sequence and simple sequence length polymorphism markers were established, of which F4I1-Sph and F4I1-Cla were the closest flanking markers identified.

(B) The *ROL5* gene consists of 10 exons encoding a protein of 355 amino acids. The G-to-A mutation in *rol5-1* is located in the second exon and changes Gly-65 to Asp. *rol5-2* represents a T-DNA insertion line that interrupts the reading frame at the amino acid codon Glu-170. Gray boxes, exons.

(C) RT-PCR experiments on RNA isolated from shoots (S) and roots (R) of 1-week-old seedlings demonstrated that the *ROL5* gene is expressed in the wild type and the *rol5-1* mutant but not to detectable levels in *rol5-2*. RT-PCR on the *ACTIN2* gene was performed to confirm the use of similar amounts of RNA in the different samples. One of two biological replicates is shown.

(D) In roots, *ROL5* is predominantly expressed in the elongation zone (ez) and in a striped pattern in the differentiation zone (dz) (top panel). A close-up of the root (GFP fluorescence in the middle panel; bright field in the bottom panel) revealed overlapping GFP fluorescence and root hair formation. Red dots, root hair–forming trichoblasts; arrow, root hair structure. Bar = 0.3 mm.

(E) When transiently expressed in *Arabidopsis* epidermal cells, ROL5-GFP (left panel) and a mitochondrial marker protein (for details, see Methods) fused to red fluorescent protein (middle panel) display overlapping fluorescence patterns (right panel). Bar = 50 μm.

of the F2 generation segregated 3:1 for *lrx1*:wild-type-like root hairs. To characterize the effect of the *rol5-1* mutation in more detail, a *rol5-1* single mutant was identified after backcrossing with wild-type Columbia (see Methods).

Map-Based Cloning of *rol5*

The *rol5* gene was identified by map-based cloning and initially localized to a region on chromosome 2, south of *AthBio2*. Further mapping revealed two flanking markers on the BAC *F4I1*, *F4I1-SphI*, and *F4I1-ClaI* at positions 16,700 and 43,500, respectively (Figure 2A). This interval was sequenced and a single point mutation was identified in the gene At2g44270 encoding a protein of 355 amino acids (Figure 2B). Transformation of the *lrx1 rol5-1* mutant with a wild-type genomic copy of this gene led to the development of an *lrx1* root hair phenotype, confirming that the identified gene represents the *ROL5* locus (Figure 1D). The mutation in *rol5-1* results in an amino acid change from Gly-65 to Asp. A *rol5* T-DNA mutant with the insertion site 3′ adjacent to the Glu-170 codon was identified and named *rol5-2* (Figure 2B). RT-PCR on RNA isolated from wild-type and mutant seedlings revealed the presence of *ROL5* RNA in wild-type and *rol5-1* mutant seedling root and shoot tissue but not in *rol5-2* seedlings (Figure 2C). Together with the position of the T-DNA in an exon, this suggests that *rol5-2* is a null allele. The *lrx1* phenotype was also suppressed by *rol5-2* (Figure 1E), revealing that suppression is not dependent on the particular missense mutation present in the *rol5-1* allele.

The RT-PCR data indicated that *ROL5* is expressed in various tissues. For a more detailed analysis, a *ROL5:ROL5-GFP* (green fluorescent protein) fusion construct was transformed into wild-type *Arabidopsis* and roots of transgenic seedlings were analyzed. Fluorescence was found to be predominant in the

Table 1. Length of Roots, Trichoblasts, and Root Hairs of the *rol5-1* Mutant

Genotype	Root Length (mm)	Epidermal Cell Length (Trichoblasts) (μm)	Root Hair Length (μm)
Wild Type	15 ± 0.2	147 ± 29	700 ± 80
rol5-1	10 ± 0.2	126 ± 24	480 ± 120

Seedlings were grown for 5 d in a vertical orientation. Values represent means ± SD. Differences are significant (*t* test, $P < 0.05$).

elongation zone and to expand in a striped pattern into the differentiation zone. These stripes overlapped with the arrangement of root hair cells (Figure 2D), which initiate root hair elongation in the differentiation zone (Dolan et al., 1994). Thus, *ROL5* is predominantly expressed in elongating cells, suggesting an important function during cell expansion. Indeed, compared with wild-type seedlings, *rol5-1* mutants had shorter roots, root epidermal cells, and root hairs (Figure 1F, Table 1).

ROL5 Is Structurally and Functionally Similar to the Yeast Ncs6p

ROL5 shows 54% identity and 70% similarity to Ncs6p/Tuc1p of yeast (*Saccharomyces cerevisiae*), subsequently referred to as Ncs6p (Figure 3). The Ncs6p-like proteins of different organisms share conserved motifs, including a PP-loop domain with ATP pyrophosphatase activity (Bork and Koonin, 1994; Björk et al., 2007), which are also conserved in ROL5. The Gly-65 to Asp mutation in *rol5-1* is adjacent to the PP-loop motif SGGxDS (Figure 3). Ncs6p-like proteins have been found to be involved in the thiolation of the uridine residue 34 of a subset of cytoplasmic tRNAs (Björk et al., 2007; Schlieker et al., 2008; Leidel et al., 2009). To investigate whether ROL5 is involved in tRNA modification in *Arabidopsis*, the tRNA fraction of wild-type and *rol5-1* mutant seedlings was isolated. tRNAs containing this modification can be detected in an acrylamide gel containing *N*-acryloylamino phenyl mercuric chloride (APM), a compound that interacts with 2-thiouridine and retards migration in the gel. While a band shift can be observed with wild-type tRNAs in gels containing APM, it is absent from *rol5-1* tRNA extracts. In the absence of APM, as expected, no shift is detectable in either of the extracts (Figure 4A). Hence, ROL5 is involved in this tRNA modification process in *Arabidopsis*.

Since Ncs6p was also identified as a component of the TOR pathway, we assessed whether Ncs6p and ROL5 have similar functions with respect to TOR signaling. The *Δncs6* mutant yeast strain, which is hypersensitive to rapamycin (Chan et al., 2000; Goehring et al., 2003a), was complemented with *ROL5* under the control of a constitutive yeast promoter. On standard growth medium, the wild type, the *Δncs6* mutant, and the complemented *Δncs6* mutant showed comparable growth properties, while in the presence of rapamycin, growth of the *Δncs6* mutant was considerably retarded. This effect was compensated for by expressing *ROL5* in the *Δncs6* mutant, but not by expressing the *rol5-1* missense allele (Figure 4B).

Ncs6p appears to accumulate in mitochondria, which prompted us to investigate the subcellular localization of ROL5. A ROL5-GFP fusion construct was transiently expressed in *Arabidopsis* epidermal cells, and colocalization with well-established organellar

Figure 3. ROL5 Is Homologous to Ncs6p of Yeast.

The alignment of ROL5 with Ncs6p of *S. cerevisiae* reveals 54% identity and 70% similarity between the two proteins. The Ncs6p-like proteins of different organisms share common motifs that are indicated below the sequences [$(CxxC)_2$ – SGGxDS – CxxC – GH – PL – C – $(CxxC)_2$], all of which are conserved between the two proteins. The motif PL is not fully conserved in Ncs6p and ROL5. Sequences important for protein activity (Björk et al., 2007) are boxed. The Gly-65 to Asp mutation in *rol5-1* (star) is adjacent to the PP-loop motif SGGxDS, which is important for ATP binding.

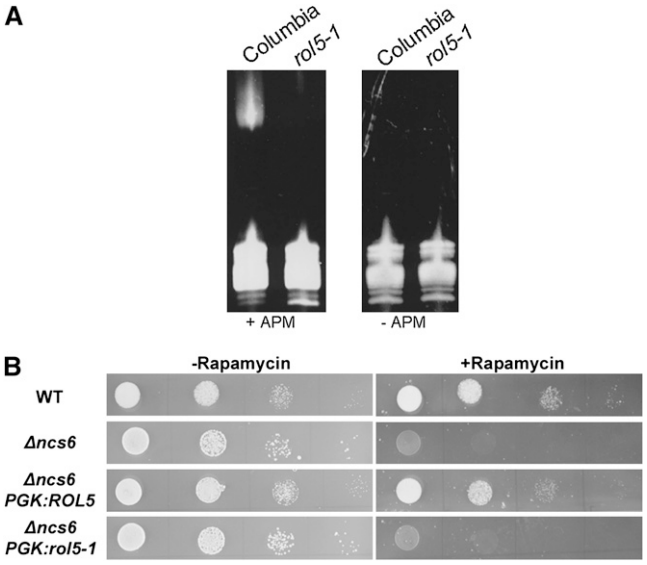

Figure 4. Ncs6p and ROL5 Have Similar Functions.

(A) tRNA was extracted from 7-d-old wild-type and *rol5-1* seedlings and separated on an acrylamide gel with (left panel) or without (right panel) APM, a compound that interacts with the 2-thiouridine and retards migration in the gel.

(B) Wild-type (WT) and *Δncs6* mutant yeast was grown in the absence or presence of rapamycin. The *Δncs6* mutant grew normally on control medium but was hypersensitive to rapamycin compared with the wild type. Expression of *ROL5* but not *rol5-1* under the control of the yeast *PHOSPHOGLYCERATE KINASE* promoter in the *Δncs6* mutant suppressed this rapamycin hypersensitivity phenotype. Spots on a line represent serial dilutions (10-fold).

marker proteins was investigated. A clear overlap was found for ROL5-GFP and a mitochondrial protein (for details, see Methods) fused to red fluorescent protein (Figure 2E). This suggests that ROL5, similar to Ncs6p in yeast, translocates to mitochondria. Together, these data demonstrate that Ncs6p and ROL5 have very similar functions in their respective organisms.

Interfering with TOR Signaling Leads to Suppression of *lrx1*

The functional similarity between ROL5 and Ncs6p suggested that the *rol5* mutant might be impaired in a TOR-related process. This led us to further investigate whether the *rol5* mutations suppress the *lrx1* root hair phenotype by influencing TOR signaling. To this end, the TOR-specific inhibitor rapamycin was used to interfere with TOR signaling in *Arabidopsis*. This required transformation of *Arabidopsis* with the yeast *FKBP12* under the control of the ubiquitously active *35S* promoter. Wild-type Columbia plants expressing *FKBP12* were produced (Figure 5A), and the *lrx1* mutation was crossed into two independent transgenic lines. While *lrx1* mutants expressing *FKBP12* developed typical *lrx1* root hairs under normal growth conditions, the presence of rapamycin led to a clear suppression of the *lrx1* phenotype in both transgenic lines. In nontransgenic *lrx1* mutants, rapamycin had no effect on the root hair phenotype (Figure 5B). This shows that interfering with TOR signaling suppresses the *lrx1* phenotype.

The treatment with rapamycin had additional effects on root development that were similar to those observed in the *rol5-1* mutant. In the presence of rapamycin, *FKBP12*-expressing wild-type plants developed shorter roots and shorter epidermal cells (Table 2) as previously observed by Sormani et al. (2007), confirming the involvement of TOR signaling in plant cell elongation.

rol5-1 and Rapamycin Treatment Lead to Changes in Cell Wall Components

A possible mechanism of suppression of the *lrx1* root hair phenotype might be through compensation of the cell wall defects in *lrx1* mutants by the introduction of additional changes to cell walls. To identify potential alterations in cell wall structures, root surfaces were analyzed in the wild type and *rol5-1* mutant using a series of monoclonal antibodies targeted to different cell wall polysaccharide components. These were antipectic homogalacturonan JIM5 and JIM7 (Knox et al., 1990), anti-(1→4)-β-D-galactan LM5 (Jones et al., 1997), anti-(1→5)-α-L-arabinan LM6 (Willats et al., 1998), antixyloglucan LM15 (Marcus et al., 2008), and anti-AGP LM2 (Yates et al., 1996). Four of these epitopes were detected at equivalent levels on wild-type and *rol5-1* mutant root surfaces (see Supplemental Figure 1 online), whereas the LM5 galactan and the LM2 AGP epitopes displayed differential modulation in response to the mutation. Detection of the LM5 galactan epitope decreased and that of the LM2 AGP epitope increased at the root surface of the

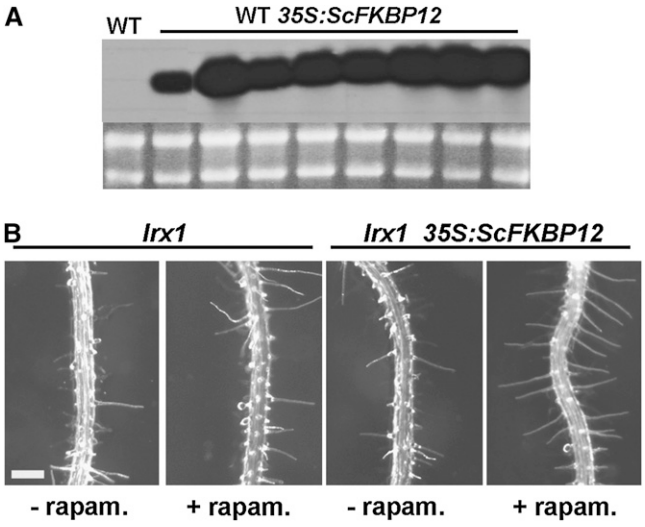

Figure 5. Rapamycin Treatment Suppresses the *lrx1* Root Hair Phenotype.

(A) RNA gel blot of wild-type (WT) *Arabidopsis* transformed with a *35S* promoter:*FKBP12* construct.

(B) *lrx1* mutants expressing *FKBP12* were sensitive to rapamycin (rapam.) and showed a suppressed *lrx1* root hair phenotype (right). In nontransgenic *lrx1* mutants, the root hair phenotype was not affected by rapamycin (left). Bar = 0.5 mm.

Table 2. Length of Roots and Trichoblasts Due to Rapamycin Treatment

35S:ScFKBP12	Root Length (mm)	Epidermal Cell Length (Trichoblasts) (μm)
− Rapamycin	15 ± 0.2	140 ± 12
+ Rapamycin	11 ± 0.2	90 ± 9

Seedlings were grown for 5 d in a vertical orientation. Values represent means ± SD. Differences are significant (*t* test, $P < 0.05$).

rol5-1 mutant (Figure 6A). The same monoclonal antibodies were used to analyze root surfaces upon interfering with TOR signaling by rapamycin. Treatment of seedlings expressing *FKBP12* with rapamycin resulted in alterations in immunolabeling that were similar to those observed in the *rol5-1* mutant. The LM5 galactan epitope showed a marked decrease in occurrence, whereas the LM2 AGP epitope was increased in proximal parts of the root (Figure 6B) compared with nontreated seedlings expressing yeast *FKBP12*. The other antibodies showed equal labeling for both conditions (see Supplemental Figure 2A online). Immunodetection with LM5 and LM2 was identical between nontransgenic wild-type seedlings grown with or without rapamycin and *FKBP12*-expressing seedlings grown without rapamycin (see Supplemental Figure 2B online). Thus, the observed modulations of these two cell wall epitopes were specifically induced by rapamycin and only in those seedlings that were expected to be rapamycin sensitive, suggesting that they were the result of impaired TOR signaling.

rol5-1 Mutants Are Affected in Their Response to ROS and ROS Scavengers

One possible crossing point of TOR signaling and ROL5 is the mitochondrial localization of ROL5, since the TOR pathway is a regulator of mitochondrial activity and hence the production of ROS. To investigate this possibility, ROS levels in roots of wild-type and *rol5-1* mutant seedlings were analyzed using different ROS-sensitive staining substrates. Under the growth conditions used, none of these stainings revealed a clear, reproducible change in ROS levels in *rol5-1* seedlings (see Supplemental Figure 3 online). Next, the effect of ROS on seedling growth was tested in liquid culture. This experiment revealed an increased susceptibility of *rol5-1* seedlings to hydrogen peroxide. While wild-type seedlings showed a similar development with or without 8 mM H_2O_2, the development of *rol5-1* seedlings was retarded in the presence of H_2O_2. By contrast, *rol5-1* seedlings were revealed to be more tolerant to ROS scavengers. While wild-type seedlings barely grew and failed to accumulate chlorophyll in the presence of 100 μm $CuCl_2$, *rol5-1* seedlings turned green and grew considerably better (Figure 7). This indicates that ROL5 is important for the sensing of, and the response to, ROS.

DISCUSSION

The work presented here suggests that the TOR pathway is a process that can lead to the specific modification of cell wall components. The *rol5* locus was identified in a suppressor screen on the *lrx1* mutant, which is affected in cell wall formation in root hairs (Baumberger et al., 2001, 2003b). This screen was performed with the aim of identifying novel loci involved in cell wall formation, as suppressors can reveal a functional relationship between genetic loci (Huang and Sternberg, 1995). After the previous identification of *rol1*, which encodes RHAMNOSE SYNTHASE1 (Diet et al., 2006), *rol5* is the second identified suppressor of *lrx1* and also affects cell wall structure.

The TOR Pathway Is a Regulator of Cell Wall Development

ROL5 is homologous to the yeast Ncs6p, and these proteins have similar functions in their respective organisms. These functions include the modification of tRNAs and the effect on TOR signaling. It is likely that suppression of *lrx1* is induced via a modification of TOR signaling, as the *lrx1* mutant root hair phenotype can be suppressed by the TOR kinase inhibitor rapamycin. Rapamycin is a macrocyclic lactone originally identified in the bacterium *Streptomyces hygroscopicus* and one of the most specific kinase inhibitors known (Heitman et al., 1991; Huang et al., 2003), making additional effects of rapamycin very unlikely. The TOR pathway is a central regulator of eukaryotic growth processes (Wullschleger et al., 2006), and previous work

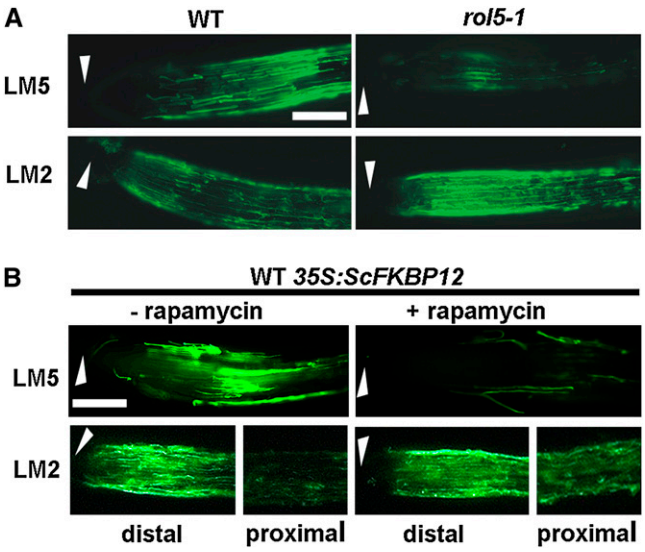

Figure 6. Immunolabeling of Cell Walls of *rol5-1* and Rapamycin-Treated Wild-Type Seedlings.

Immunolabeling of 4-d-old roots with monoclonal antibodies (1→4)-β-D-galactan side chains of RG I (LM5) and glucuronic acid–containing side chains of arabinogalactan proteins (LM2).

(A) Compared with the wild type (WT), the *rol5-1* mutant root surface revealed reduced detection of the LM5 epitope and stronger detection of the LM2 epitope.

(B) Roots of *FKBP12*-expressing wild-type seedlings grown in the absence (left) and presence (right) of rapamycin. The presence of rapamycin led to a reduced labeling with LM5 and a stronger labeling in proximal parts with LM2.

Arrowheads, root apex. Bars = 0.3 mm.

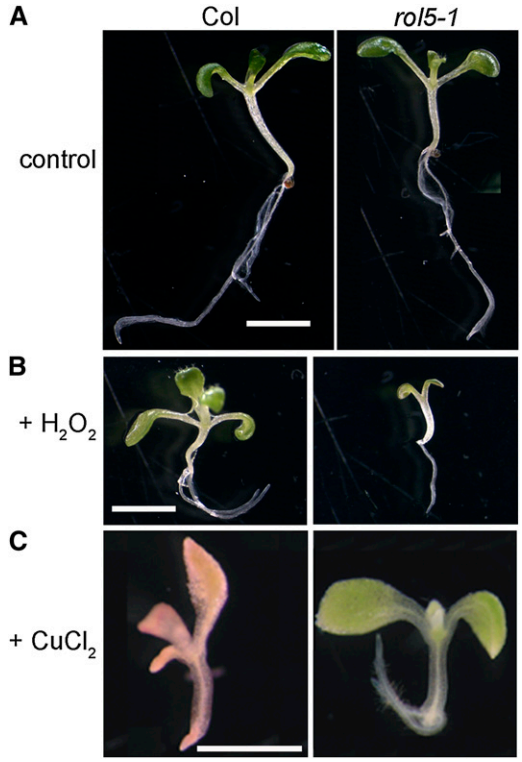

Figure 7. Altered Response of the *rol5-1* Mutant to ROS and ROS Scavenger.

Seedlings were grown in liquid culture for 10 d. Under control conditions **(A)**, growth of the wild type and *rol5-1* is comparable. *rol5-1* seedlings are hypersensitive to H_2O_2 (8 mM), revealed by the reduced growth **(B)**, and hyposensitive to the ROS scavenger $CuCl_2$ (100 μm), indicated by better growth and the development of green cotyledons **(C)**. col, wild-type Columbia.

in *Arabidopsis* has revealed the importance of this signaling pathway, including the TOR kinase itself, for plant development (Menand et al., 2002; Bögre et al., 2003; Mahfouz et al., 2006). Our analysis shows that the TOR pathway is able to modify cell wall components, suggesting that it is part of the regulatory process that coordinates cell wall structure with cell growth and development. In fact, recent work in *C. albicans* suggests that the TOR pathway is involved in sensing cell wall integrity. The *rhb1* mutant, affected in a small G-protein of the RAS superfamily, was found to be hypersensitive to drugs interfering with cell wall formation and showed a modified cell wall integrity signaling previously identified in this organism. The *rhb1* mutation induces rapamycin hypersensitivity in *C. albicans*, indicating a function of RHB1 in TOR signaling (Tsao et al., 2009). Plants clearly have mechanisms to monitor, sense, and modify cell wall composition and structures. Proteins involved in this process have been found to be transmembrane or membrane associated, such as wall-associated kinases, the receptor kinase THESEUS, GPI-anchored proteins, or lectins (Kohorn et al., 2006; Hematy et al., 2007; Humphrey et al., 2007; Hematy and Höfte, 2008). These proteins are probably directly involved in the regulatory or sensing process, since they localize to cell surfaces. Considering the importance in regulating cell growth, the TOR pathway might function as a relay system that integrates the signals of sensing mechanisms into cellular responses and developmental processes.

The core component of the TOR pathway is the Ser/Thr kinase protein TOR. Only yeast encodes two separate but functionally similar TOR proteins. In yeast and mammals, TOR forms two distinct multiprotein complexes, TORC1 and TORC2, of which only TORC1 is rapamycin sensitive. While TORC1 is involved in regulating translation, nutrient import, or stress responses, TORC2 influences the actin cytoskeleton (Loewith et al., 2002; Wullschleger et al., 2006). Also in *Arabidopsis*, the diverse functions of this pathway are likely to require the establishment of distinct multiprotein complexes with the TOR protein. The TOR-interacting proteins RICTOR and RAPTOR were identified in TORC1 and TORC2 (Wullschleger et al., 2006), and RAPTOR has been shown to undergo interactions with the *Arabidopsis* TOR protein. Mutations in RAPTOR affect plant development, corroborating the importance of this protein for the TOR pathway (Deprost et al., 2005; Mahfouz et al., 2006). It is likely that TOR-interacting proteins establish further protein–protein interactions as shown for other organisms (Wullschleger et al., 2006), leading to diverse signaling outputs. A better understanding of the signaling network is required to identify the mechanism by which the TOR pathway senses and influences cell wall structures.

Interfering with TOR Modifies Cell Walls and Plant Growth

Plant cell walls are complex, structurally variable organelles that underpin many aspects of cell and organ growth. Cell wall extension is the limiting factor in cell enlargement (Carpita and Gibeaut, 1993; Martin et al., 2001). Monoclonal antibodies are a useful tool to analyze cell walls as they can detect changes in the composition or the accessibility of cell wall structures. The LM5 galactan epitope that is a component of RGI has been specifically detected in the elongation zone at the *Arabidopsis* root surface and implicated in the onset of the acceleration of cell elongation (McCartney et al., 2003). The occurrence of the LM5 epitope has also been observed to correlate with modified mechanical properties of cell walls and to be reduced in different mutants that show reduced root epidermal cell growth (McCartney et al., 2000, 2003; Diet et al., 2006). Thus, the short root phenotype observed in the *rol5-1* mutant and in rapamycin-treated seedlings correlates well with reduced LM5 labeling and the known involvement of this cell wall component with growth. Pectin regulates the porosity of cell walls and hence influences the mobility of cell wall modifying enzymes necessary for cell wall expansion (Baron-Epel et al., 1988). RGI is thought to modify this porosity, which would serve as a possible explanation of why changes in the RGI structure influence cell growth (Ridley et al., 2001; Willats et al., 2001). AGPs are a second cell wall component found to be altered due to modified TOR signaling. The LM2 antibody, which binds to a glucuronic acid–containing epitope of AGPs (Yates et al., 1996), showed increased immunolabeling of *rol5-1* mutants and rapamycin-treated seedlings. The modified distribution and/or abundance of AGPs has been shown to correlate with aberrant cell growth in roots, root hairs, and pollen tubes as demonstrated by the analysis of *agp* mutants as well as the use of Yariv reagent

that precipitates AGPs and blocks their action (Willats and Knox, 1996; Ding and Zhu, 1997; van Hengel and Roberts, 2002; McCartney et al., 2003; Levitin et al., 2008). Reducing root epidermal cell expansion with Yariv reagent also modifies the occurrence of the LM5 epitope, indicating some linkage between RGI and AGPs (McCartney et al., 2003) that has also now been shown in the *rol5-1* mutant and rapamycin-treated seedlings. The reduced cell elongation phenotypes observed in the *rol5* mutants and upon rapamycin treatment therefore correlate with changes to specific cell wall components. Thus, the TOR pathway might be a regulatory mechanism to modulate these two factors in cell walls. It is possible that the observed changes in root surface detection of the LM5 galactan and LM2 AGP epitopes are mechanistically involved in the suppression of the *lrx1* root hair phenotype. As *lrx1* mutants develop aberrant cell walls, it can be hypothesized that secondary modifications overcome the defects induced by the lack of LRX1.

ROL5 Might Have Dual Functions

It remains unclear exactly how ROL5 affects TOR signaling. The *rol5-1* mutant, similar to the yeast *Δncs6* mutant, fails to properly modify tRNAs. The uridine residue 34 of several tRNAs is modified to 5-methoxycarbonylmethyl-2-thiouridine to improve translational efficiency. Ncs6p, together with other proteins, has been shown to transfer the sulfur group during 2-thiouridine formation (Björk et al., 2007; Schlieker et al., 2008; Leidel et al., 2009; Noma et al., 2009). The TOR pathway is involved in regulating the translational machinery in different organisms, including plants (Mahfouz et al., 2006; Wullschleger et al., 2006; Dinkova et al., 2007), and the lack of tRNA modifications might trigger signals that feed back into TOR signaling. This indirect effect on the TOR pathway is a possible explanation for the rapamycin hypersensitivity of the *Δncs6* mutant (Chan et al., 2000; Goehring et al., 2003a). Alternatively, Ncs6p and ROL5 might have an additional, so far unidentified function that links protein activity to the TOR signaling network. Indicative for this hypothesis is the localization of these proteins to mitochondria (Huh et al., 2003; this work). Previous work has shown that Ncs6p is dispensable for the thiolation of mitochondrial tRNA (Noma et al., 2009), suggesting an additional function of the protein in this organelle. The dual localization in different compartments has been shown for a number of proteins (Krause and Krupinska, 2009). Goehring et al. (2003a) reported on the influence of Ncs6p on protein conjugation by Urm1p, a ubiquitin-related modifier protein. Yeast Urm1p has recently been shown to be involved in the same sulfur carrier process as Ncs6p (Leidel et al., 2009). In addition, however, Urm1p is also conjugated to Ahp1p, which is not involved in tRNA modification but is likely to have a function in TOR signaling (Goehring et al., 2003b). Our analysis points to an additional effect of ROL5 in a ROS-related process as the *rol5-1* mutant showed an increased sensitivity to ROS and an increased tolerance to ROS scavengers compared with the wild type. The mitochondrial localization of ROL5 is consistent with this additional function since mitochondria are a major source of ROS (Rhoads et al., 2006). ROS are not just byproducts of the respiratory chain but revealed to serve as signaling molecules that can affect cell elongation and cell wall development (Liszkay et al., 2004; Takeda et al., 2008). ROL5 might regulate the response of the cell to ROS signaling, which is in agreement with the reduced cell growth observed in *rol5-1* mutants. A major function of the TOR pathway is the regulation of mitochondrial activity (Schieke et al., 2006; Cunningham et al., 2007), and ROL5 might be part of this regulatory mechanism.

The TOR pathway is a central regulator of eukaryotic cell growth. The analyses presented here suggest that the TOR pathway has the ability to modify cell wall structure and specifically components implicated in cell elongation. The TOR pathway appears to be one mechanism of connecting plant cell growth processes with specific changes to cell wall structure. Further analyses are necessary to identify the proteins that establish the link between the TOR signaling network and the extracellular proteins that sense and survey cell wall developmental processes. Moreover, the possible multiple activities of ROL5-like proteins need to be elucidated in greater detail to identify their precise roles during cell growth.

METHODS

Plant Growth, EMS Mutagenesis, and Mapping

The *lrx1* missense allele and the EMS mutagenesis procedure are described by Diet et al. (2004). The *lrx1* mutant and all other *Arabidopsis thaliana* lines used are in the Columbia genetic background, except for the line used for mapping, which is Landsberg *erecta* (L*er*). The *rol5-2* allele (line 709D04) was obtained from the GABI collection (Rosso et al., 2003). Phenotypic analysis was performed on *lrx1 rol5-1* and *rol5-1* mutant plants backcrossed twice with the *lrx1* mutant and wild-type Columbia, respectively. *lrx1 rol5-1* and *lrx1 rol5-2* double mutants and *rol5-1* single mutants were identified with molecular markers for the mutations (see below). For growth of plants in sterile conditions, seeds were surface sterilized with 1% sodium hypochlorite and 0.03% Triton X-100, stratified for 3 d at 4°C, and grown for 5 d on half-strength Murashige and Skoog (MS) medium containing 0.6% Phytagel (Sigma-Aldrich), 2% sucrose, and 100 mg/L myo-inositol, or, for liquid culture, in half-strength MS medium, 1% sucrose, and 100 mg/L myo-inositol, with a 16-h-light/8-h-dark cycle at 22°C. For crosses and propagation of the plants, seedlings were transferred to soil and grown in growth chambers with a 16-h-light/8-h-dark cycle at 22°C. Plant transformation was performed as described by Diet et al. (2006).

For mapping, the *lrx1 rol5-1* mutant was crossed with L*er* and propagated to the F2 generation. Five hundred F2 seedlings displaying a wild-type root hair phenotype were selected and screened for homozygous *lrx1* mutant plants with a PCR-based marker (Diet et al., 2004). These plants were assumed to be homozygous mutants for *rol5-1* and were thus used for initial mapping. Once the approximate map position of *rol5* was identified, F2 plants displaying an *lrx1* mutant phenotype (i.e., being homozygous mutant *lrx1*) were selected, and those heterozygous Columbia/L*er* in the region containing the *rol5* locus were propagated to the F3 generation. As expected, seedlings of the F3 population segregated 3:1 for *lrx1* versus wild-type root hairs. One thousand wild-type-like F3 seedlings were selected for detailed mapping of *rol5*. Mapping was performed using standard simple sequence length polymorphism and cleaved-amplified polymorphic sequence markers developed based on the Columbia/L*er* polymorphism databank (Jander et al., 2002).

Molecular Markers for Genotyping

The marker for *lrx1* was previously described (Diet et al., 2004). The *rol5-1* mutation was detected by PCR with the primers *rol5BanI_F*

(5′-ACAATCTTAAGAGGCAAACC-3′) and *rol5BanI_R* (5′-CATATTAAG-CAGAAGCTTGG-3′), followed by digestion with the enzyme *Ban*I, which only cuts wild-type *ROL5* but not the *rol5-1* DNA. The T-DNA insertion in *rol5-2* is 3′ adjacent to the sequence 5′-GTTATTGAAAGTAGAGA-3′. Homozygous *rol5-2* mutants were identified by DNA gel blotting using genomic DNA digested with *Bgl*II and a fragment of the *ROL5* gene 3′ adjacent of the T-DNA insertion site as a specific probe for hybridization.

DNA Constructs

For complementation of the *rol5-1* mutant, a *ROL5* genomic clone including 1.8 kb of the promoter region and 400 bp of terminator sequence was amplified by PCR using the primer pair *Rol5R1Not* (5′-ATTGCGGCC-GCTGGGCTGGTGATGAAAGTTG-3′), *Rol5F1NotI* (5′-ATTGCGGCCGC-CAGAGTGTCTTGATTGGTTCG-3′). The PCR product was digested by *Not*I and cloned into the *pART27* plant transformation vector (Gleave, 1992) cut with the same enzyme. For the ROL5-GFP fusion constructs, the genomic clone of *ROL5* that was used for complementation was subjected to site-directed mutagenesis (QuikChange; Stratagene) to introduce one *Bam*HI site using the primer pair *mutBamHI-midF* (5′-GAATCTCCTCCT-CGGATCCAAAAACCTCATAAAAGC-3′) and *mutBamHI-midR* (5′-GCTTT-TATGAGGTTTTTGGATCCGAGGAGGAGATTC-3′). The *GFP* gene was amplified from the vector *pMDC83* (Curtis and Grossniklaus, 2003) using the primer pair *GFP-F* (5′-TATGGATCCATGAGTAAAGGAGAAGAACT-TTTC-3′), *GFP-R* (5′-AATGGATCCGT-GGTGGTGGTGGTGGTGTTTG-3′) and cloned into the *Bam*HI site of the *ROL5* gene. The resulting *ROL5-GFP* construct was cloned into the binary vector *pART27* with the restriction enzyme *Not*I. For transient expression in *Arabidopsis* epidermal cells, the *ROL5-GFP* construct was ligated into the overexpression cassette of pART7 (Gleave, 1992) and used for particle bombardment. CoxIV-DsRed with a yeast COXIV presequence tag for mitochondrial localization (Mollier et al., 2002) was used as the mitochondrial marker protein. The overexpression construct for Sc *FKBP12* was obtained by PCR amplification of *FKBP12* from yeast with the primer pair *ScFKBP12_F* (5′-GAATTCATGTCTGAAGTAATTGAAGGTAAC-3′), *ScFKBP12_R* (5′-TCTAGATTAGTTGACCTTCAACAATTCGAC-3′) and cloning of the PCR product into *pART7* containing a *35S cauliflower mosaic virus promoter:ocs terminator* cassette (Gleave, 1992) by digestion with *Eco*RI and *Xba*I. A correct *pART7-ScFKBP12* clone was digested with *Not*I, and the excised *35S:ScFKBP12:ocs* cassette was inserted into the binary vector *pART27* digested with *Not*I. For expression in yeast, the coding sequence of a *ROL5* cDNA was amplified with the primers *ROL5-pFL61_F* (5′-GCGGCCGCATGGAGGCCAAGAACAA-GAAAGC-3′) and *ROL5-pFL61_R* (5′-GCGGCCGCTTAGAAATCCAGA-GATCCACATTG-3′) and cloned into the expression vector *pFL61* (Minet et al., 1992) by digestion with *Not*I.

Yeast Strain and Growth Conditions

Yeast strains used in this study were obtained from EUROSCARF, Frankfurt, Germany. The wild-type strain is BY4741 with the relevant genotype MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0, and the ncs6Δ strain has the relevant genotype MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YGL211w::kanMX4. Yeast strains were grown using standard methods. Synthetic yeast media was prepared with 2.4 nM rapamycin where indicated. Yeast strains were grown at 30°C until log phase and drops of an OD_{600} of 0.8 and three subsequent 10-fold dilutions were spotted onto synthetic solid medium and grown for 3 d at 30°C.

Transient Gene Expression in *Arabidopsis* Epidermal Cells

For transient gene expression, *Arabidopsis* leaf epidermal cells were transformed by particle bombardment as described (Escobar-Restrepo et al., 2007). Bombarded tissue was incubated for 2 d at room temperature and the fluorescence analyzed using confocal microscopy.

ROS Staining

For ROS staining, 0.1 mg/mL NBT was directly dissolved in 0.1 M K-phosphate buffer, pH 7, stirred for 60 min at room temperature, and filtered through a 0.2-μm pore size filter. Seedlings were incubated at room temperature for 60 min. The reaction was stopped by washing twice with 100% ethanol.

Microscopy

GFP fluorescence was analyzed by confocal microscopy (DMIRE2; Leica) and analysis of immunolabeling on a LM510 (Zeiss). Phenotypic observations were performed with a Leica LZ M125 stereomicroscope. For cell and root hair length measurements, pictures were taken by differential interference contrast microscopy using an Axioplan microscope (Zeiss). Over 30 data points from ≥5 seedlings were collected. Root length was manually determined, using ≥20 seedlings per data point. The *t* test was used for statistical analysis, and the values are given with ± SD, P = 0.05. Confocal microscopy was performed on a DMIRE2 (Leica).

Immunolabeling

Immunolabeling of surfaces of intact *Arabidopsis* seedling roots was performed using six rat monoclonal antibodies directed to cell wall components. *Arabidopsis* seedlings were prepared for immunofluorescence microscopy as described (McCartney et al., 2003). Seedlings were vertically grown for 4 d prior to immunolabeling. An FITC-linked anti-rat antibody (Sigma-Aldrich) was used as secondary antibody. Seedlings were mounted in a glycerol antifade solution (Citifluor AF1; Agar Scientific) for microscopy observation.

RNA Extraction and RT-PCR

Seedlings were grown vertically on half-strength MS plates for 2 weeks. Approximately 150 seedlings of each plant line were cut at the hypocotyl to separate shoot and root tissue, and the tissues were used for extraction of total RNA using the TRIzol method (Gibco BRL). The reverse transcription was performed using the SuperScriptTM II RNase reverse transcriptase kit (Invitrogen). The resulting single-stranded DNA was used for PCR with 30 cycles. *ACTIN2* was amplified as a control using the primer pair *Actin2F* (5′-AATGAGCTTCGTATTGCTCC-3′) and *Actin2R* (5′-GCACAGTGTGAGACACACC-3′). Levels for the *ROL5* transcript were checked using the primer pair *Rol5NorthF3* (5′-CCAAGATGTAAACCTTT-CAAG-3′) and *RolNorth2R* (5′-GCTTCTTTGTTTCCTTATTATG-3′).

tRNA Extraction and Analysis

Whole seedlings grown for 14 d in half-strength liquid medium containing 1% sucrose and 100 mg/L myo-inositol were collected for tRNA extraction. Plant material was frozen in liquid nitrogen, ground, and stored at −80°C. Total RNA was extracted two times with acidic phenol and chloroform and one time with chloroform. tRNA was purified using Nucleobond AX 100 columns (Macherey-Nagel) and precipitated overnight at −20°C. One microgram of tRNA per sample was separated on an 8% polyacrylamide gel containing 7 M urea and 1 μg/μL APM chloride where indicated. For tRNA visualization, the gel was stained with SYBR Gold (Invitrogen).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession

numbers: *ROL5*, At2G44270; *LRX1*, At1G12040; Sc *FKBP12*, YNL023C; and Sc *NCS6*, YGL211W.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Cell Wall Epitopes Not Affected by the *rol5-1* Mutation.

Supplemental Figure 2. Effect of Rapamycin on Cell Wall Epitopes.

Supplemental Figure 3. ROS Staining in Wild-Type and *rol5-1* Mutant Roots.

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REFERENCES

Anderson, G.H., Veit, B., and Hanson, M.R. (2005). The Arabidopsis *AtRaptor* genes are essential for post-embryonic plant growth. BMC Biol. **3:** 12.

Baron-Epel, O., Gharyal, P.K., and Schindler, M. (1988). Pectins as mediators of wall porosity in soybean cells. Planta **175:** 389–395.

Baumberger, N., Doesseger, B., Guyot, R., Diet, A., Parsons, R.L., Clark, M.A., Simmons, M.P., Bedinger, P., Goff, S.A., Ringli, C., and Keller, B. (2003a). Whole-genome comparison of leucine-rich repeat extensins in Arabidopsis and rice: A conserved family of cell wall proteins form a vegetative and a reproductive clade. Plant Physiol. **131:** 1313–1326.

Baumberger, N., Ringli, C., and Keller, B. (2001). The chimeric leucine-rich repeat/extensin cell wall protein LRX1 is required for root hair morphogenesis in *Arabidopsis thaliana*. Genes Dev. **15:** 1128–1139.

Baumberger, N., Steiner, M., Ryser, U., Keller, B., and Ringli, C. (2003b). Synergistic interaction of the two paralogous Arabidopsis genes *LRX1* and *LRX2* in cell wall formation during root hair development. Plant J. **35:** 71–81.

Björk, G.R., Huang, B., Persson, O.P., and Bystrom, A.S. (2007). A conserved modified wobble nucleoside (mcm(5)s(2)U) in lysyl-tRNA is required for viability in yeast. RNA **13:** 1245–1255.

Bögre, L., Okresz, L., Henriques, R., and Anthony, R.G. (2003). Growth signalling pathways in Arabidopsis and the AGC protein kinases. Trends Plant Sci. **8:** 424–431.

Bork, P., and Koonin, E.V. (1994). A p-loop-like motif in a widespread ATP pyrophosphatase domain: Implications for the evolution of sequence motifs and enzyme-activity. Proteins **20:** 347–355.

Carpita, N.C., and Gibeaut, D.M. (1993). Structural models of primary cell walls in flowering plants: Consistency of molecular structure with the physical properties of the walls during growth. Plant J. **3:** 1–30.

Chan, T.F., Carvalho, J., Riles, L., and Zheng, X.F.S. (2000). A chemical genomics approach toward understanding the global functions of the target of rapamycin protein (TOR). Proc. Natl. Acad. Sci. USA **97:** 13227–13232.

Cunningham, J.T., Rodgers, J.T., Arlow, D.H., Vazquez, F., Mootha, V.K., and Puigserver, P. (2007). mTOR controls mitochondrial oxidative function through a YY1-PGC-1 alpha transcriptional complex. Nature **450:** 736–740.

Curtis, M.D., and Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. Plant Physiol. **133:** 462–469.

Deprost, D., Truong, H.N., Robaglia, C., and Meyer, C. (2005). An Arabidopsis homolog of RAPTOR/KOG1 is essential for early embryo development. Biochem. Biophys. Res. Commun. **326:** 844–850.

Deprost, D., Yao, L., Sormani, R., Moreau, M., Leterreux, G., Nicolai, M., Bedu, M., Robaglia, C., and Meyer, C. (2007). The Arabidopsis TOR kinase links plant growth, yield, stress resistance and mRNA translation. EMBO Rep. **8:** 864–870.

Diet, A., Brunner, S., and Ringli, C. (2004). The *enl* mutants enhance the *lrx1* root hair mutant phenotype of *Arabidopsis thaliana*. Plant Cell Physiol. **45:** 734–741.

Diet, A., Link, B., Seifert, G.J., Schellenberg, B., Wagner, U., Pauly, M., Reiter, W.D., and Ringli, C. (2006). The *Arabidopsis* root hair cell wall formation mutant *lrx1* is suppressed by mutations in the *RHM1* gene encoding a UDP-L-rhamnose synthase. Plant Cell **18:** 1630–1641.

Ding, L., and Zhu, J.K. (1997). A role for arabinogalactan-proteins in root epidermal cell expansion. Planta **203:** 289–294.

Dinkova, T.D., de la Cruz, H.R., Garcia-Flores, C., Aguilar, R., Jimenez-Garcia, L.F., and de Jimenez, E.S. (2007). Dissecting the TOR-S6K signal transduction pathway in maize seedlings: Relevance on cell growth regulation. Physiol. Plant. **130:** 1–10.

Dolan, L., Duckett, C.M., Grierson, C., Linstead, P., Schneider, K., Lawson, E., Dean, C., Poethig, S., and Roberts, K. (1994). Clonal relationships and cell patterning in the root epidermis of Arabidopsis. Development **120:** 2465–2474.

Escobar-Restrepo, J.M., Huck, N., Kessler, S., Gagliardini, V., Gheyselinck, J., Yang, W.C., and Grossniklaus, U. (2007). The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception. Science **317:** 656–660.

Gapper, C., and Dolan, L. (2006). Control of plant development by reactive oxygen species. Plant Physiol. **141:** 341–345.

Gleave, A.P. (1992). A versatile binary vector system with a T-DNA organizational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Mol. Biol. **20:** 1203–1207.

Goehring, A.S., Rivers, D.M., and Sprague, G.F. (2003a). Urmylation: A ubiquitin-like pathway that functions during invasive growth and budding in yeast. Mol. Biol. Cell **14:** 4329–4341.

Goehring, A.S., Rivers, D.M., and Sprague, G.F. (2003b). Attachment of the ubiquitin-related protein Urm1p to the antioxidant protein Ahp1p. Eukaryot. Cell **2:** 930–936.

Heitman, J., Movva, N.R., and Hall, M.N. (1991). Targets for cell-cycle arrest by the immunosuppressant rapamycin in yeast. Science **253:** 905–909.

Hematy, K., and Höfte, H. (2008). Novel receptor kinases involved in growth regulation. Curr. Opin. Plant Biol. **11:** 321–328.

Hematy, K., Sado, P.E., Van Tuinen, A., Rochange, S., Desnos, T., Balzergue, S., Pelletier, S., Renou, J.P., and Höfte, H. (2007). A receptor-like kinase mediates the response of Arabidopsis cells to the inhibition of cellulose synthesis. Curr. Biol. **17:** 922–931.

Huang, L.S., and Sternberg, P.W. (1995). Genetic dissection of developmental pathways. In Methods in Cell Biology, H.F. Epstein and C.C. Shaker, eds (San Diego, CA: Academic Press), pp. 97–122.

Huang, S., Bjornsti, M.A., and Houghton, P.J. (2003). Rapamycins. Cancer Biol. Ther. **2:** 222–232.

Huh, W.K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S., and O'Shea, E.K. (2003). Global analysis of protein localization in budding yeast. Nature **425:** 686–691.

Humphrey, T.V., Bonetta, D.T., and Goring, D.R. (2007). Sentinels at the wall: Cell wall receptors and sensors. New Phytol. **176:** 7–21.

Ingram, G.C., and Waites, R. (2006). Keeping it together: Co-ordinating plant growth. Curr. Opin. Plant Biol. **9:** 12–20.

Jander, G., Norris, S.R., Rounsley, S.D., Bush, D.F., Levin, I.M., and Last, R.L. (2002). Arabidopsis map-based cloning in the post-genome era. Plant Physiol. **129:** 440–450.

Jones, L., Seymour, G.B., and Knox, J.P. (1997). Localization of pectic galactan in tomato cell walls using a monoclonal antibody specific to (1->4)-beta-D-galactan. Plant Physiol. **113:** 1405–1412.

Knox, J.P., Linstead, P.J., King, J., Cooper, C., and Roberts, K. (1990). Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. Planta **181:** 512–521.

Kohorn, B.D., Kobayashi, M., Johansen, S., Riese, J., Huang, L.F., Koch, K., Fu, S., Dotson, A., and Byers, N. (2006). An Arabidopsis cell wall-associated kinase required for invertase activity and cell growth. Plant J. **46:** 307–316.

Krause, K., and Krupinska, K. (2009). Nuclear regulators with a second home in organelles. Trends Plant Sci. **14:** 194–199.

Leidel, S., Pedrioli, P.G.A., Bucher, T., Brost, R., Costanzo, M., Schmidt, A., Aebersold, R., Boone, C., Hofmann, K., and Peter, M. (2009). Ubiquitin-related modifier Urm1 acts as a sulphur carrier in thiolation of eukaryotic transfer RNA. Nature **458:** 228–232.

Levitin, B., Richter, D., Markovich, I., and Zik, M. (2008). Arabinogalactan proteins 6 and 11 are required for stamen and pollen function in Arabidopsis. Plant J. **56:** 351–363.

Liszkay, A., van der Zalm, E., and Schopfer, P. (2004). Production of reactive oxygen intermediates ($O_2^{\cdot-}$, H_2O_2, and $^{\cdot}OH$) by maize roots and their role in wall loosening and elongation growth. Plant Physiol. **136:** 3114–3123.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P., and Hall, M.N. (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol. Cell **10:** 457–468.

Mahfouz, M.M., Kim, S., Delauney, A.J., and Verma, D.P.S. (2006). *Arabidopsis* TARGET OF RAPAMYCIN interacts with RAPTOR, which regulates the activity of S6 kinase in response to osmotic stress signals. Plant Cell **18:** 477–490.

Majewska-Sawka, A., and Nothnagel, E.A. (2000). The multiple roles of arabinogalactan proteins in plant development. Plant Physiol. **122:** 3–9.

Marcus, S.E., Verhertbruggen, Y., Herve, C., Ordaz-Ortiz, J.J., Farkas, V., Pedersen, H.L., Willats, W.G.T., and Knox, J.P. (2008). Pectic homogalacturonan masks abundant sets of xyloglucan epitopes in plant cell walls. BMC Plant Biol. **8:** 60.

Martin, C., Bhatt, K., and Baumann, K. (2001). Shaping in plant cells. Curr. Opin. Plant Biol. **4:** 540–549.

McCartney, L., Ormerod, A.P., Gidley, M.J., and Knox, J.P. (2000). Temporal and spatial regulation of pectic (1->4)-beta-D-galactan in cell walls of developing pea cotyledons: Implications for mechanical properties. Plant J. **22:** 105–113.

McCartney, L., Steele-King, C.G., Jordan, E., and Knox, J.P. (2003). Cell wall pectic (1 -> 4)-β-D-galactan marks the acceleration of cell elongation in the Arabidopsis seedling root meristem. Plant J. **33:** 447–454.

Menand, B., Desnos, T., Nussaume, L., Berger, F., Bouchez, D., Meyer, C., and Robaglia, C. (2002). Expression and disruption of the Arabidopsis TOR (target of rapamycin) gene. Proc. Natl. Acad. Sci. USA **99:** 6422–6427.

Minet, M., Dufour, M.E., and Lacroute, F. (1992). Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. Plant J. **2:** 417–422.

Mollier, P., Hoffmann, B., Debast, C., and Small, I. (2002). The gene encoding *Arabidopsis thaliana* mitochondrial ribosomal protein S13 is a recent duplication of the gene encoding plastid S13. Curr. Genet. **40:** 405–409.

Noma, A., Sakaguchi, Y., and Suzuki, T. (2009). Mechanistic characterization of the sulfur-relay system for eukaryotic 2-thiouridine biogenesis at tRNA wobble positions. Nucleic Acids Res. **37:** 1335–1352.

Rhoads, D.M., Umbach, A.L., Subbaiah, C.C., and Siedow, J.N. (2006). Mitochondrial reactive oxygen species. Contribution to oxidative stress and interorganellar signaling. Plant Physiol. **141:** 357–366.

Ridley, B.L., O'Neill, M.A., and Mohnen, D.A. (2001). Pectins: Structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry **57:** 929–967.

Ringli, C. (2005). The role of extracellular LRR-extensin (LRX) proteins in cell wall formation. Plant Biosyst. **139:** 32–35.

Rosso, M.G., Li, Y., Strizhov, N., Reiss, B., Dekker, K., and Weisshaar, B. (2003). An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol. Biol. **53:** 247–259.

Schieke, S.M., and Finkel, T. (2006). Mitochondrial signaling, TOR, and life span. Biol. Chem. **387:** 1357–1361.

Schieke, S.M., Phillips, D., McCoy, J.P., Aponte, A.M., Shen, R.F., Balaban, R.S., and Finkel, T. (2006). The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. J. Biol. Chem. **281:** 27643–27652.

Schlieker, C.D., Van der Veen, A.G., Damon, J.R., Spooner, E., and Ploegh, H.L. (2008). A functional proteomics approach links the ubiquitin-related modifier Urm1 to a tRNA modification pathway. Proc. Natl. Acad. Sci. USA **105:** 18255–18260.

Sormani, R., Yao, L., Menand, B., Ennar, N., Lecampion, C., Meyer, C., and Robaglia, C. (2007). *Saccharomyces cerevisiae* FKBP12 binds *Arabidopsis thaliana* TOR and its expression in plants leads to rapamycin susceptibility. BMC Plant Biol. **7:** 26.

Takeda, S., Gapper, C., Kaya, H., Bell, E., Kuchitsu, K., and Dolan, L. (2008). Local positive feedback regulation determines cell shape in root hair cells. Science **319:** 1241–1244.

Tsao, C.C., Chen, Y.T., and Lan, C.Y. (2009). A small G protein Rhb1 and a GTPase-activating protein Tsc2 involved in nitrogen starvation-induced morphogenesis and cell wall integrity of *Candida albicans*. Fungal Genet. Biol. **46:** 126–136.

van Hengel, A.J., and Roberts, K. (2002). Fucosylated arabinogalactan-proteins are required for full root cell elongation in Arabidopsis. Plant J. **32:** 105–113.

Willats, W.G.T., and Knox, J.P. (1996). A role for arabinogalactan-proteins in plant cell expansion: Evidence from studies on the interaction of beta-glucosyl Yariv reagent with seedlings of *Arabidopsis thaliana*. Plant J. **9:** 919–925.

Willats, W.G.T., Marcus, S.E., and Knox, J.P. (1998). Generation of a monoclonal antibody specific to (1->5)-alpha-L-arabinan. Carbohydr. Res. **308:** 149–152.

Willats, W.G.T., McCartney, L., Mackie, W., and Knox, J.P. (2001). Pectin: Cell biology and prospects for functional analysis. Plant Mol. Biol. **47:** 9–27.

Wullschleger, S., Loewith, R., and Hall, M.N. (2006). TOR signaling in growth and metabolism. Cell **127:** 5–19.

Yates, E.A., Valdor, J.F., Haslam, S.M., Morris, H.R., Dell, A., Mackie, W., and Knox, J.P. (1996). Characterization of carbohydrate structural features recognized by anti-arabinogalactan-protein monoclonal antibodies. Glycobiology **6:** 131–139.

4.2 Ubiquitin-related modifiers of Arabidopsis function in tRNA modification and protein conjugation

Running head: Ubiquitin-related modifiers of Arabidopsis

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Ubiquitin-related modifiers of *Arabidopsis thaliana* function in tRNA modification and protein conjugation

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ABSTRACT

The Ubiquitin-related modifier (Urm) proteins belong to the class of ubiquitin-like proteins (UBLs). UBLs do not share high sequence homology to Ubiquitins but they all share a β-grasp motif as typical structural feature. A number of UBL proteins exist in eukaryotes, where they do not influence protein turnover but serve other functions. In this work, we functionally characterize the Urm homologs of *Arabidopsis thaliana*, URM1 and URM2. In yeast and humans, Urm proteins are known to be essential for the thiolation of tRNAs. This process is important for the efficiency and accuracy of protein translation. Here we show that in Arabidopsis, the *urm1 urm2* double mutant does not allow for the production of thiolated tRNAs. Furthermore the yeast *Δurm1* mutant can be complemented with either *URM1* or *URM2*. This suggests that URM1 and URM2 are the functional and structural Arabidopsis homologs of yeast Urm1p and thus genuine Urm proteins. Apart from being essential for tRNA thiolation, evidence accumulated that in yeast and human fibroblasts, Urms are attached to proteins in response to cellular oxidative-stress. The data presented here suggest that the Arabidopsis Urms are also conjugated to other proteins. Since this process is observed even under non-stressed conditions, the Arabidopsis Urms, in contrast to those of yeast and human cells, appear to have a function in basic physiological processes of the plant.

INTRODUCTION

Posttranslational modification of proteins adds an additional level of diversification in structure of proteins which can affect their activity, stability, or location. One of these modifications is the attachment of polypeptides such as ubiquitin (Ub) or related proteins to lysine residues of target proteins. Ubiquitylation is the reversible attachment of Ub to proteins involving activation, conjugation, and ligation of Ub via corresponding E1, E2, and E3 ligase

activities, respectively (Kerscher et al., 2006). While polyubiquitylation targets proteins to degradation via the proteasome, single ubiquitylation has non-proteolytic effects on cellular processes such as transcription, chromatin modifications, or vesicle dynamics. In addition to ubiquitin, a number of ubiquitin-like modifiers are present in most eukaryotes that are also able to tag proteins usually in a transient manner (Hochstrasser, 2009; Park et al., 2011; Vierstra, 2012). In a number of non-plant eukaryotic systems, ubiquitin-related modifiers (URMs) were identified that are not highly homologous to ubiquitin in respect to the amino acid sequence but share a β-grasp motif as typical structural feature of this type of protein. URMs are particular in that they were initially identified as serving a role as sulfur carriers in tRNA thiolation (Furukawa et al., 2000; Xu et al., 2006; Schlieker et al., 2008; Leidel et al., 2009; Noma et al., 2009). This process involves activation of URM by a sulfurtransferase with E1-like activity which adenylates URMs and transfers sulfur to the terminal glycine resulting in a thiocarboxylate. With the activity of thiouridylases, Nsc2p and Ncs6p in yeast or CTU2 and ATPBD3 in humans, the thiol group is then transferred onto uridine residues of a small group of tRNAs, a modification which is thought to increase translation efficiency (Björk et al., 2007). The enzymes with E2- and E3-like activity assumed to be necessary have so far not been identified (Van der Veen et al., 2011). The function of URMs in thiolation of tRNAs is reminiscent of sulfur transfer reactions in prokaryotes in the synthesis of molybdopterin and thiamine. The MoaD/ThiS proteins involved in this process are not homologous in sequence to URMs, yet also show the β grasp motif (Kessler, 2006). In archaea, the Urm-like proteins SAMP1 and SAMP2 are involved in sulfur transfer including thiolation of tRNAs (Miranda et al., 2011). Hence, URM type proteins appear to have an activity different from other ubiquitin-related proteins and, because of their similarity to prokaryotic sulfur transfer systems, are considered to be evolutionary intermediates between prokaryotic sulfur transfer and eukaryotic ubiquitin-like protein conjugation systems (Wang et al., 2011). In addition to the established role of URM proteins in tRNA thiolation, there is increasing evidence for a second role of URMs in urmylation, a protein modification similar to

ubiquitylation in which URMs are conjugated to lysine of target proteins (Goehring et al., 2003a; Van der Veen et al., 2011).

The tRNA modification process in plants has not yet been thoroughly investigated. ROL5, the Arabidopsis homolog of Ncs6p and ATPBD3 of yeast and humans, respectively, is a protein experimentally shown to be involved in thiolation of tRNAs in plants. *rol5* mutant plants show a slight growth retardation and are sensitive to oxidative stress (Leiber et al., 2010). So far, experimental evidence is missing for the existence of other components of the tRNA thiolation machinery, Urm-like proteins, and protein urmylation in Arabidopsis, even though Urm-homologous genes are encoded in the Arabidopsis genome.

The Arabidopsis genome encodes two Urm-like proteins. URM1 and URM2 were characterized genetically, in respect to their activity, as well as for protein –protein interaction. This study reveals that both proteins are functionally comparable to the yeast Urm1p, are involved in tRNA modification, and interact with the same protein as their yeast homolog. Hence, the function of Urm-like proteins is well-conserved across phylogenetically distantly related species such as archaea, yeast, mammals, and plants. In addition, genetic analysis revealed that the URM proteins influence plant development. One mode of action may be urmylation which, in contrast to yeast or humans, is observed in Arabidopsis under normal growth conditions.

RESULTS

Arabidopsis possesses two proteins with high sequence and functional similarity to yeast Urm1p

Ubiquitin related modifier-like (URM) proteins have been identified in a number of organisms. Blast search of the Arabidopsis genome for proteins related to URM1p of yeast (*Saccharomyces cerevisiae*) revealed two proteins encoded in the Arabidopsis genome with high similarity. The proteins encoded by the At2g45695 and At3g61113 (referred to as *URM1* and *URM2*, respectively) share 35% and 39% identity, and 63% and 65% similarity, respectively, with ScUrm1p. Also the human Urm1 shows a high degree of homology (53% and 54% identity to URM1 and URM2, respectively, and 76% similarity to both proteins), indicating that this type of protein is well conserved across distantly related species. The C-terminal half of the proteins show a higher degree of identity, suggesting that this moiety is less tolerant to variations in amino acid sequence. In particular the terminal diglycine motif essential for Urm protein1 function is also present in URM1 and URM2 (Fig. 1A). The two URM proteins of Arabidopsis share high homology to each other with an identity of 87% and a similarity of 91% (Fig. 1B).

Figure 1 Conservation of URM proteins among different species. A, Alignment of URM1 and URM2 of *Arabidopsis thaliana* with URM1p of *Saccharomyces cerevisiae* and URM1 of *Homo sapiens* reveals strong similarity between the URM proteins of these three species, particularly in the C-terminal moiety. B, Alignment of the Arabidopsis URM1 and URM2 show a high degree of identity.

In a next step, it was tested whether URM1 and/or URM2 have a function in the sulfur carrier process important for the thiolation of eukaryotic cytoplasmic transfer RNAs (tRNAs) as shown for yeast and human Urm proteins. To this end, complementation of the yeast Δ*urm1* mutant defective in tRNA thiolation (Leidel et al., 2009) was used as the experimental system. The Δ*urm1* mutant was transformed with the cDNAs of the Arabidopsis *URM1* or *URM2* under the control of a constitutive active yeast promoter. These complementation strains were then analyzed for the presence of thiolated tRNAs. The binding of N-acryloylamino phenyl mercuric chloride (APM) to 2-thiouridine residues leads to the retardation of thiolated tRNAs in acrylamide gels, making thiolated tRNAs readily detectable. The thiolated tRNAs detectable in wild-type yeast but absent in the Δ*urm1* mutant are present in the Δ*urm1* mutant complemented with either *URM1* or *URM2* (Fig. 2). Complementation with constructs encoding N-terminal GFP-URM proteins also led to

thiolation, i.e. complementation of the Δ*urm1* mutant phenotype, indicating that an N-terminal GFP, considerably bigger in size than the URM proteins, does not interfere with URM protein function (Fig. 2). This result demonstrates that the Arabidopsis URM1 andtURM2 are functionally similar to the yeast Urm1p.

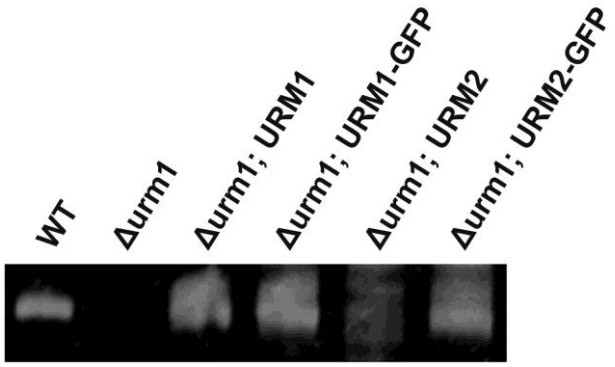

Figure 2 URM1, URM2, and Urm1p have similar activities in tRNA thiolation. Bulk tRNA was extracted from wild-type (WT*)*, *Δurm1* and *Δurm1* yeast strains complemented with *URM1* or *URM2*. Thiolated tRNAs show slower migration in an acrylamide gel containing APM. Only slower migrating tRNAs are shown. URM proteins and the GFP-URM fusion proteins of Arabidopsis are functional in yeast, resulting in tRNA thiolation in the otherwise thiolation-defective *Δurm1* mutant.

Urm1p has been shown to localize to the cytoplasm and to the nucleus (Ghaemmaghami et al., 2003). The subcellular localization of the Arabidopsis URM proteins was investigated with the GFP-URM fusion proteins. In both cases, a strong cytoplasmic and nuclear GFP fluorescence was detectable (Fig. 3, A and B). In addition, a *GFP-URM1* construct was transiently transformed into Arabidopsis seedlings and onion epidermis cells. Also in these tissues, GFP fluorescence was detected in the cytoplasm and in the nucleus (Fig. 3, C and D), indicating that subcellular localization is conserved in different organisms.

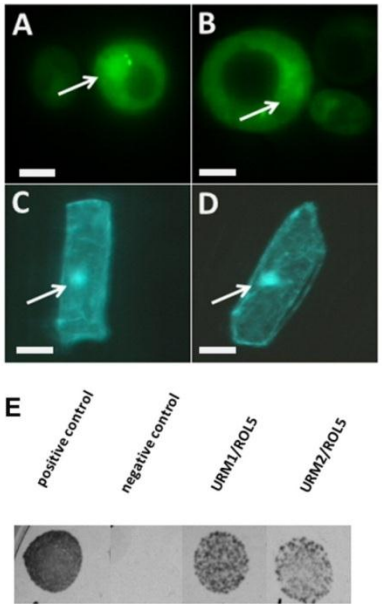

Figure 3 Arabidopsis URM proteins localize to the cytoplasm and the nucleus and interact with ROL5. Transient expression of an N-terminal GFP-URM1 (A) and GFP-URM2 (B) fusion in yeast cells results in cytoplasmic and nuclear florescence. A comparable localization is found for GFP-URM1 in Arabidopsis (C) or onion (D) epidermal cells. E, Yeast-two-hybrid experiment verified the interaction of URM1 and URM2 with ROL5. Bars = 5µm (A and B), 50 µm (C and D).

Arabidopsis URM proteins interact with ROL5

The protein network of yeast leading to tRNA thiolation has been investigated in detail. Within this network, Urm1p interacts with the thiouridylase Ncs6p (Schlieker et al., 2008; Leidel et al., 2009). To get an insight into the degree of conservation of this network in Arabidopsis, URM1 and URM2 were tested for interaction with ROL5, the Arabidopsis thiouridylase and functional homolog of Ncs6p (Leiber et al., 2010). To this end, a yeast-two-hybrid experiment was performed using ROL5 as the bait protein and either URM1 or URM2 as the prey protein. For both URM proteins, an interaction with ROL5 was observed (Fig. 3E). The utilized yeast-two-hybrid constructs were tested negative for auto-activation activity,

excluding false positive results. This provides further evidence for the conservation of the process of sulfur transfer leading to tRNA modification across a wide range of species.

***URM1* and *URM2* are ubiquitously expressed**

According to microarray data of the Genevestigator platform (Zimmermann et al., 2004), *URM1* and *URM2* are expressed at all developmental stages of Arabidopsis. To investigate in which tissue types *URM1* and *URM2* are expressed, the promoter sequences of *URM1* and *URM2* were fused to the *GUS* gene and transformed into Arabidopsis. These *GUS* expressing lines were then screened for GUS activity at seedling and adult stage. At the seedling stage, both *URM1* and *URM2* promoter induced *GUS* expression in most tissues, leading to a blue staining of the entire seedlings (Fig. 4) that was stronger in the vasculature. In adult plants, GUS activity was found in most tissues, with *URM1-GUS* resulting in a stronger GUS staining than *URM2-GUS*, which is in agreement with microarray data that found *URM1* to be expressed at a higher level (Zimmermann et al., 2004). Again, staining was particularly strong in the vascular tissue (Fig. 4). The overall similarity of the expression pattern of *URM1* and *URM2* suggests that they are largely redundant in their expression pattern.

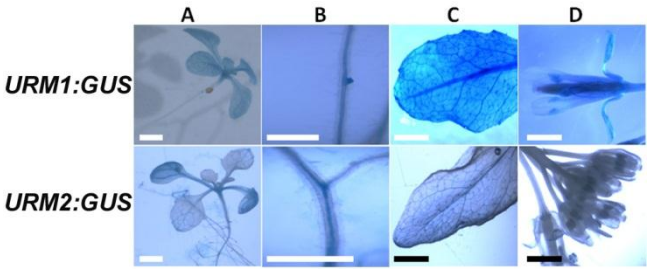

Figure 4 *URM1* and *URM2* are expressed in similar tissues. The expression pattern of both genes was investigated by promoter-*GUS* fusion constructs in transgenic Arabidopsis. Both constructs led to a homogeneous GUS staining at the seedling stage and the adult stage. Shoots (A) and roots (B) of seedlings; cauline leaves and stems (C) and flowers (D) of adult plants. Bars = 2.5 mm

Mutations in the *URM* genes affect plant physiology and development

To determine the significance of *URM1* and *URM2* for plant development, T-DNA insertion lines of these loci were identified. For *URM1*, one insertion line (*urm1-1*) was identified which contains a T-DNA insertion in the first intron. For *URM2*, two insertion lines were identified, *urm2-1* harbors the insertion 250 bp upstream of the start codon and *urm2-2* in the first intron (Fig. 5A). RT-PCR experiments on total RNA extracted from homozygous mutants revealed that both *urm1-1* and *urm2-1* still produced *URM1* and *URM2* mRNAs, respectively, that are identical in sequence with the wild-type RNA. At the other hand, the lack of an *URM2* RT-PCR product on total RNA of the *urm2-2* mutant shows that expression level is strongly affected (Fig. 5B). However, quantitative RT-PCR on RNA extracted from wild-type and *urm1-1* mutant seedlings showed that the level of *URM1* RNA is strongly reduced in *urm1-1* compared to the wild type, making *urm1-1* a knock-down allele of *URM1* (Fig. 5C).

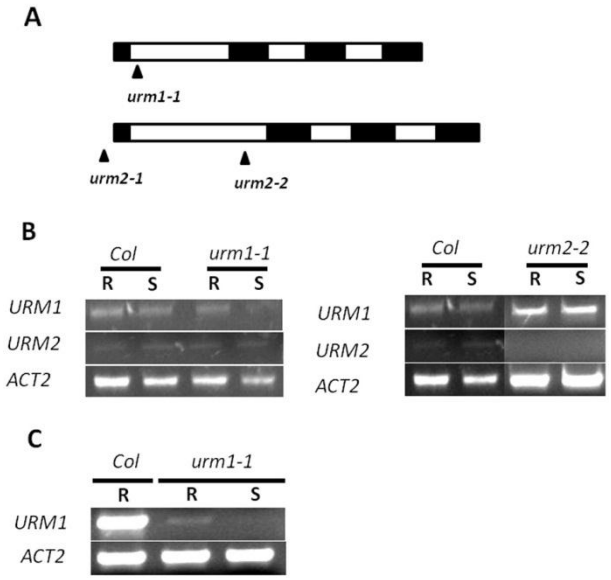

Figure 5 Gene expression in *urm1* and *urm2* T-DNA mutants. A, Schematic sequence structure of *URM1* and *URM2*. Black boxes represent exons and white boxes introns. T-DNA insertions are highlighted by black arrows and are located in the first intron for *urm1-1* and *urm2-2*, which were further analyzed. B, RT-PCR on root and shoot material of *urm1-1* and *urm2-2* seedlings. *urm1-1* plants still produce *URM1* mRNA in the shoot and in the root. In contrast *urm2-2* plants have no detectable expression level of *URM2*. The *ACTIN2* gene was amplified as quantitative standard. C, Semi quantitative RT-PCR on wild-type and *urm1-1* RNA revealed strongly reduced gene expression in the *urm1-1* allele.

To test whether these mutant alleles have an effect on tRNA thiolation, tRNAs were isolated from wild-type and mutant plants and analyzed. As a negative control, the *rol5-1* mutant was used that was previously shown to lack thiolated tRNAs (Leiber et al., 2010). A shifted tRNA band, i.e. thiolated tRNAs, were observed only in the wild type but neither in *rol5-1* nor in two independently identified *urm1-1 urm2-2* double mutants. Hence, the remaining expression level in the *urm1-1* allele appears insufficient to provide for a detectable level of thiolated tRNAs (Fig. 6A).

When growing the *urm1-1* and *urm2-2* single or *urm1-1 urm2-2* double mutants, no obvious growth defects could be observed. Since Urm-like proteins have been shown to be induced by oxidative stress (Van der Veen et al., 2011), the single and double mutants were also grown in the presence of the ROS H_2O_2 or the ROS scavenger $CuCl_2$. Also under these stress conditions, however, no aberrant developmental effect was observed in the mutant lines compared to the wild type.

The locus coding for the URM1 and URM2-interacting protein ROL5 was previously identified as a suppressor of *lrx1*, a cell wall formation mutant affected in root hair development (Leiber et al., 2010). For this reason, the ability of the *urm* mutations to suppress *lrx1* was assessed. Wild-type plants develop regular root hairs which are malformed or absent in the *lrx1* mutant. While the *urm1-1* mutation had only a slight effect on *lrx1* and *urm2-2* did not alter the *lrx1* phenotype, an *lrx1 urm1-1 urm2-2* triple mutant showed a clear suppression of *lrx1*. Root hairs frequently formed in a manner comparable to the wild type (Fig. 6B). The synergistic interaction of the two *urm* mutations provide further evidence for *URM1* and *URM2* having similar functions, which is not only seen on the level of tRNA modification but also in cell development.

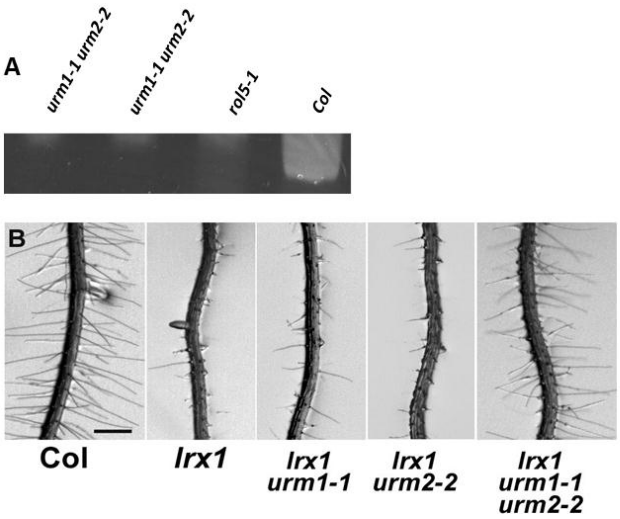

Figure 6 Effects of *urm1-1 urm2-2* on tRNA metabolism and plant development. A, Thiolated tRNAs, retarded in their migration, are present in the wild type but not in the negative control *rol5-1* or in two independently isolated *urm1-1 urm2-2* double mutant lines. B, Compared to the wild type, *lrx1* mutants develop aberrant root hairs. The *lrx1* phenotype is only partially or not suppressed by *urm1-1* and *urm2-2*, respectively. The *lrx1 urm1-1 urm2-2* triple mutant shows suppression of *lrx1* and develops wild type-like root hairs. Bar = 5 mm.

URM proteins function in urmylation

Initially, Urm-like proteins were thought to be involved in tRNA thiolation but it was controversial whether or not they also serve in the covalent attachment and thus tagging (urmylation) of other proteins. To test whether in Arabidopsis, URM1 and URM2 are conjugated to proteins, constructs coding for hemagglutinin (HA)-tagged URM fusion proteins (HA-URM) under the control of the constitutive 35S promoter were transformed into Arabidopsis. Since N-terminal GFP-URM fusion proteins are active in yeast (Fig. 2), it is reasonable to assume that HA-URM proteins are also functional. Western blots using an anti-HA antibody on total protein extracts of transgenic lines revealed expression of the fusion proteins (Fig. 7). In a next step, HA-URM proteins were immunopurified from plant

extracts with an anti-HA antibody and isolated proteins were separated by SDS-PAGE and detected by western blotting using an anti-HA antibody. Several bands of varying intensity present only in extracts of transgenic but not wild-type plants were detected. These bands had a much larger mass than the 12 kDa calculated for the monomeric forms of HA-URMs and thus are considered to represent proteins that are covalently attached to HA-URMs. Hence, these results suggest that URM proteins are not only involved in tRNA modification but also urmylate other proteins.

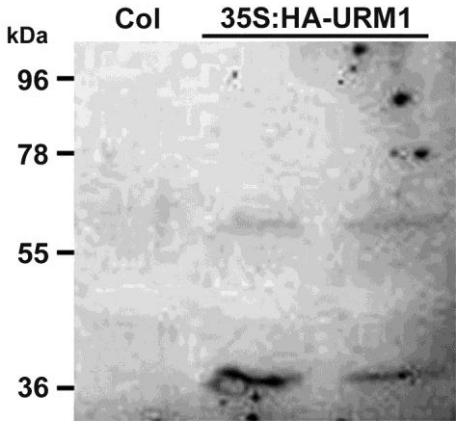

Figure 7 Urmylation of Arabidopsis proteins. Plant extracts of wild-type and two independent transgenic lines expressing HA-URM1 were used for protein precipitation using an anti-HA antibody. SDS-PAGE followed by western blotting using an anti-HA antibody revealed several proteins accumulating only in the transgenic lines but not the wild type, representing URM1-conjugated (urmylated) proteins.

DISCUSSION

Ubiquitin-related modifier proteins (Urms) are found in phylogenetically distantly related species. While their function and mode of action has recently been characterized in archaea , yeast, and human cells, little is known about Urm-like proteins in plants. *Arabidopsis* encodes two *URM* genes that seem to have very similar if not identical activities, and are both ubiquitously expressed. URM proteins of distantly related species can have relatively low level of identity or similarity, as found for Arabidopsis URM1 and URM2 versus yeast Urm1p (35% and 39%, respectively), but are conserved in the β-grasp, a characteristic structure consisting of a core with a pocket of four β-strands and diagonally arranged α-helices (Kerscher et al., 2006; Park et al., 2011; Vierstra, 2012). This structural conservation allows both Arabidopsis *URM* genes to complement the tRNA modification phenotype of the yeast Δ*urm1* mutant. Hence, most if not all Urm-like proteins seem involved in the thiolation of the uridine in the wobble position of specific tRNAs (Schlieker et al., 2008; Leidel et al., 2009; Miranda et al., 2011). URM proteins are involved in the sulfur carrier reaction. They are activated by an E1-ligase, leading to a transfer of sulfur to the terminal glycine and subsequently associate with a thiouridylase which mediates thiolation of the uridines (Leidel et al., 2009). The E1 ligase necessary for activation of URM proteins is Uba4p in yeast and in Arabidopsis is likely encoded by the gene previously identified as *SIR1*/*CNX5* (Zhao et al., 2003; Teschner et al., 2010). The Arabidopsis ROL5 is the homolog of the yeast thiouridylase Ncs6p both of which have been shown to be essential for tRNA modification (Björk et al., 2007; Schlieker et al., 2008; Leidel et al., 2009; Leiber et al., 2010). The interaction of the URM proteins with ROL5 confirms that also this part of the protein interaction network required for tRNA modification is well-conserved in Arabidopsis.

As found for Ub (Ubiquitin) and UBLs (Ubiquitin-related modifiers), URM proteins are covalently attached to other proteins, referred to as urmylation. In contrast to Ub and canonical UBLs, however, URM proteins are conjugated to Lys residues of their target

proteins via a terminal thiocarboxylate (Van der Veen et al., 2011). Posttranslational modifications are a way to often transiently alter protein activity, localization, or transport, and over two hundred different types of protein modifications are known (Kerscher et al., 2006; Park et al., 2011; Vierstra, 2012). In yeast and human cells, urmylation seems particularly abundant under oxidative stress (Goehring et al., 2003a; Van der Veen et al., 2011). In Arabidopsis, however, urmylation seems not dependent on oxidative stress, suggesting that this type of protein modification is part of the basic physiology. The biological relevance of urmylation remains unclear. In HeLa cells, proteins of the tRNA thiolation process are urmylated, possibly as an internal feet-back loop (Van der Veen et al., 2011). In yeast, the oxidative stress response protein Ahp1p has been reported to be urmylated in response to oxidative stress (Goehring et al., 2003a). Ahp1p is a peroxiredoxin and catalyzes the decomposition of reactive oxygen species (Lian et al., 2012), but the effect of urmylation on Ahp1p remains to be determined. Similar to Ub or UBLs, urmylation might affect processes such as protein activity, localization, or turnover. In fact, there is evidence for urmylation being a reversible process (Goehring et al., 2003a; Petroski et al., 2011; Van der Veen et al., 2011), making it unlikely that targeting proteins for degradation is a main purpose of urmylation.

The thiolation of the uridine in the wobble position of specific tRNAs is assumed to increase codon-anticodon accuracy (Björn et al., 2007). The absence of this modification, however, seems not essential under normal growth conditions. Neither yeast *Δurm1* mutants (Furukawa et al., 2000; Goering et al., 2003b) nor the Arabidopsis *urm1-1 urm2-2* double mutant missing detectable levels of thiolated tRNAs seem impaired in growth. Even though data from other systems indicate a function of URM proteins in oxidative stress response (Goehring et al., 2003a; Van der Veen et al., 2011) the Arabidopsis *urm1-1 urm2-2* double mutant did not show aberrant phenotypes under stress conditions. Hence, under these stress conditions and under non-stressed conditions, neither this particular type of tRNA modification nor the urmylation of proteins appear important. It remains to be shown under

which circumstances or stages of plant development URM proteins are crucial. Since *urm1-1* is a knock-down rather than a knock-out allele, a sufficient amount of URM1 might be produced in the *urm1-1* mutant to prevent a strong growth phenotype. Despite this possibility, the amount of URM1 protein is reduced in the *urm1-1* mutant, since *urm1-1* and *urm2-2* function synergistically in suppressing the *lrx1* root hair growth phenotype. The individual *urm1-1* or *urm2-2* mutants are not sufficient for suppression of *lrx1*, providing further evidence that the two genes are functionally redundant. *lrx1* is a root hair mutant that is affected in cell wall formation (Baumberger et al., 2001; 2003; Ringli, 2005). Previous analyses revealed that mutating the thiouridylase ROL5, i.e. blocking tRNA thiolation, causes suppression of the *lrx1* phenotype (Leiber et al., 2010). The comparable effect by the *urm1-1 urm2-2* double mutant suggests that interfering with tRNA modification is causing this suppression. The absence of tRNA thiolation does affect the TOR (target of rapamycin) signaling network, a major controller of cell growth in eukaryotes that regulates a plethora of processes including translation (Chan et al., 2000; Goehring et al. 2003b; Wullschleger et al., 2006). It is conceivable that modifications in translational efficiency caused by the absence of tRNA thiolation (Björk et al., 2007) induce alterations in TOR signaling. The *rol5* mutation affects TOR signaling in Arabidopsis and inhibiting TOR by rapamycin (Huang et al., 2003) is sufficient to induce suppression of *lrx1* (Leiber et al., 2010; John et al., 2011). Together, these data suggest that interfering with tRNA modification triggers signaling to the TOR pathway, which in turn modifies cell growth. In addition, the altered extent of urmylation caused by the *urm1-1 urm2-2* double mutant is likely to also affect TOR signaling. Proteins of the tRNA thiolation process have been shown to be targets of urmylation (Van der Veen et al., 2011) and changes in the urmylation pattern might affect TOR signaling.

The data presented here support the view that the tRNA thiolation process is highly conserved across distantly related species. This assumption is corroborated by the interaction of the yeast Urm1p with Ncs6p that is also found for their Arabidopsis equivalents URM1 and URM2 with ROL5. This requires an overlapping localization of the two proteins in

the cell. Protein localization studies revealed that the Arabidopsis URMs and the yeast Urm1p (Goehring et al., 2003a) localize to the cytoplasm. Their interaction partners ROL5 and Ncs6p, respectively, appear to be predominantly accumulating in mitochondria (Huh et al., 2003; Leiber et al., 2010). Based on the function of Ncs6p and ROL5, however, it is likely that they are, at least transiently, also present in the cytoplasm (Goehring et al., 2003b; Leiber et al., 2010) and dual localization of proteins in different compartments is not unusual (Krause and Krupinska, 2009). Hence, ROL5 and Ncs6p both appear to be mobile proteins that translocate to some extend between mitochondria and the cytoplasm where they interact with URM proteins.

The analysis of the URM proteins of Arabidopsis revealed that they are very similar in function to their homologs in yeast or humans and thus, URMs represent evolutionarily conserved proteins. Even though tRNA thiolation conferred by URMs is not essential for plant survival, this process does affect plant development. In future experiments, it will be interesting to identify the growth condition(s) that require protein urmylation. The characterization of the target proteins of urmylation should then shed light on the biological significance of urmylation in plants and possibly other organisms.

MATERIALS AND METHODS

DNA constructs

For complementation of the yeast *Δurm1* mutant, cDNA clones of the Arabidopsis *URM1* and *URM2* were amplified using the primer pairs *URM1*_for(GGATCCATGCAATTAACTCTTGAATTCGGG) / *URM1*_rev(TTATCCACCATGCAAAGTGGAAAT) and *URM2*_for (GGATCCATGCAATTTACTCTTGAGTTCGGT) / *URM2*_rev (TCATCCACCGTGCAGAGTCGAAAT). The obtained fragments were cloned into pGEM-T-easy (Promega) for sequencing. For obtaining the N-terminal GFP fusions, the correct cDNAs in pGEM-T easy constructs were digested with *Bam*HI and a *GFP-Bam*HI cassette (Leiber et al., 2010) was inserted. The resulting clones were digested with *Not*I and cloned into the yeast overexpression vector pFL61 (Minet et al., 1992).

For the yeast two-hybrid experiment, cDNAs of *ROL5*, *URM1* and *URM2* were amplified with the primer pairs *Kpn*I-*At2g44270*-1F(GGTACCATGGAGGCCAAGAACAAGAAAGCAG)/*Sma*I-*At2g44270*-1R(CCCGGGTTAGAAATCCAGAGATCCACATTG) for *ROL5* , *Xba*I-*URM1*-F (TCTAGAATGCAATTAACTCTTGAATTCG)/ *Bam*HI-*URM1*-R(GGATCCTTATCCACCATGCAAAGTGGAA) for *URM1* and *Xba*I-*URM2*-F(TCTAGAATGCAATTTACTCTTGAGTTCGGTGGAG)/*Bam*HI-*URM2*R (GGATCCTCATCCACCGTGCAGAGTCGAAATGAAA) for *URM2*. These fragments were then cloned into pGEM-T-easy for sequencing. Subsequently, one of the *ROL5* clones was digested with *Kpn*I and *Sac*I and cloned into pLEXA-N (Dualsystems) cut with the same enzymes. The clones of *URM1* and *URM2* were digested with *Xba*I and *BamH*I and cloned into pGAD-HA (Dualsystems) cut with the same enzymes.

For expressing HA-tagged versions of *URM1* and *URM2*, genomic clones were amplified using the primer pairs *URM1_HA*_gen_for (CTCGAGATGTACCCATACGATGTTCCAGATTACGCTATGCAATTAACTCTTGAATTCGG GTAC)/*URM1_HA*_gen_rev (TCTAGAGAAAGGACACTTAAAATTGATAAATACTCTAATATCA) and *URM2_HA*_gen_for (CTCGAGATGTACCCATACGATGTTCCAGATTACGCTATGCAATTTACTCTTGAGTTCGG GTACACTATTAC)/ *URM2_HA*_gen_rev(TCTAGA TCATCCACCGTGCAGAGTCGAAATGAAAACTATA), respectively. For sequencing, the clones were ligated into pGEM-T-easy. Correct clones were digested with *Xho*I and *Xba*I and cloned into the expression cassette of pART7 (Gleave, 1992) cut with the same enzymes. The N-terminal genomic *GFP* fusion construct of *URM1* was produced by *Xho*I digestion of *pART7-HA-URM1* and insertion of an *Xho*I-*GFP* cassette. The expression constructs were then cut with *Not*I and cloned into plant transformation vector pBART (Stintzi and Browse, 2000) which is identical to pART27 (Gleave, 1992) but contains a basta instead of a kanamycin resistance gene. The *GFP* gene was amplified from the vector pMDC83 (Curtis and Grossniklaus, 2003) using the primer pair *GFP_Xho*I_for(CTCGAGATGAGTAAAGGAGAAGAACTTTTC) and *GFP_Xho*I_rev_NOSTOP(CTCGAGGTGGTGGTGGTGGTGGTGTTT).

Plant material and growth conditions

All plant lines used are of the accession Columbia. The *lrx1* allele is described in Diet et al. (2004). The *urm1-1* and *urm2-2* allele are the Salk lines 024513 and 070672.47.90. The *urm2-1* allele is the line ET5108 and is of the accession Landsberg *erecta*.

Seedlings and plants were grown *in vitro* and in soil as described by Leiber et al. (2010). In brief, seeds were surface sterilized, washed and grown in a vertical orientation with a 16-h-light/8-h-darkcycle at 22^{o}C on plates containing half-strength MS medium. For further growth

and propagation, seedlings were transferred to soil and grown with a 16-h-light/8-h-dark cycle at 22^{o}C.

Yeast strains and growth conditions

Yeast strains used in this study were obtained from EUROSCARF, Frankfurt, Germany. The wild-type strain is BY4741 with the relevant genotype MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0, and the *Δurm1* strain has the relevant genotype BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YIL008w::kanMX4. Yeast strains were grown at 30°C for 2 d on SD plates supplemented with His, Leu, Ade for strains complemented with *pFL61* constructs and His, Leu, Ade and Ura for growth of the wild type.

Transient gene expression in Arabidopsis epidermal cells

For transient gene expression, Arabidopsis leaf epidermal cells were transformed by particle bombardment as described (Escobar-Restrepo et al., 2007). Bombarded tissue was incubated for 1 d at room temperature and the fluorescence pattern was microscopically analyzed.

Microscopy

Epidermal GFP fluorescence was analyzed using a Zeiss Imager Z1 microscope equipped with an Axiocam HRC. GFP fluorescence of yeast cells was analyzed with a Leica DM6000 equipped with a Leica BFC 350FX. Phenotypic observations and *GUS* expression analysis were done with a Leica LZ M125 stereomicroscope.

RNA extraction and RT-PCR

Seedlings were grown vertically on half-strength MS Medium in a vertical orientation for 14 d as described above. Shoot and root tissue of 120 seedlings separated, frozen in liquid nitrogen, and grinded. The powder was then used for RNA extraction using a SV Total RNA isolation System kit (Promega). The reverse transcription was conducted using the M-MLV Reverse Transcriptase RNase H Minus kit (Promega). The obtained cDNA was then used for RT-PCR using the primer pairs *ACTIN2*F (59-AATGAGCTTCGTATTGCTCC-39) and *ACTIN2*R (59GCACAGTGTGAGACACACC-39), *URM1*_rt_for(ATGCAATTAACTCTTGAATTCG) / *URM1*_rt_rev(TTATCCACCATGCAAAGTGGAA), and *URM2*_rt_for(ATGCAATTTACTCTTGAGTTCG)/ *URM2*_rt_rev(TCATCCACCGTGCAGAGTCGAA). For semi-quantitative analysis of *URM1* expression, the same primer pair was used with 25 PCR cycles.

tRNA extraction and analysis

Arabidopsis seedlings were grown vertically on plates for 14 d as described. Approximately 250 seedlings were used for extraction. The seedlings were grinded in liquid nitrogen and the material was extracted two times with 8ml acidic phenol (Sigma), 0.8ml chloroform and once with 4ml acidic phenol, 0.4ml chloroform.

Yeast strains were grown at 30°C in 50ml liquid SD media supplemented with His, Leu, Ade for strains complemented with *pFL61* constructs and His, Leu, Ade and Ura for growth of the wild type or *Δurm1* mutant. The tRNA was extracted 2 times with 4ml acidic phenol, 0.4ml chloroform.

After extraction of the plant or yeast material, tRNA was purified with AX100 columns from MACHEREY NAGEL following manufacturer's instructions. For analysis, the purified tRNA

was separated on an acrylamide gel supplemented with N-acryloylamino phenyl mercuric chloride (APM) by the method adapted from Björk et al. (2007).

Immunoprecipitation and Western blotting

For immunoprecipitation, plant material was grinded in liquid nitrogen and extracted with 10% glycerol, 50 mM Tris pH7.5, 150 mM NaCl, 0.1%Nonidet P40, 1 tablet complete Protease Inhibitor Cocktail (Roche) per 10ml of buffer on ice. After two rounds of centrifugation at +4^{o}C, sepharose-coupled rat anti HA-antibody (Roche) was added to the clear supernatant and incubated for 1 h at +4^{o}C in an overhead shaker. The sepharose was pelleted by centrifugation and washed twice with 20 mM Tris pH7.5, 0.1M NaCl, 0.1 mM EDTA, 0.05% Tween20, prior to denaturation of the precipitated proteins with SDS-PAGE loading buffer.

SDS-PAGE and western blotting was performed using semi-dry blotting and standard incubation procedures with rabbit anti-HA antibody (Santa Cruz Biotechnology) as the primary and peroxidase-coupled goat anti-rabbit antibody (Sigma) as the secondary antibody, at a 1:3000 dilution each.

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LITERATURE CITED

Baumberger N, Ringli C, Keller B (2001) The chimeric leucine-rich repeat/extensin cell wall protein LRX1 is required for root hair morphogenesis in Arabidopsis thaliana. Genes Dev **15:** 1128-1139

Baumberger N, Steiner M, Ryser U, Keller B, Ringli C (2003) Synergistic interaction of the two paralogous Arabidopsis genes *LRX1* and *LRX2* in cell wall formation during root hair development. Plant J **35:** 71-81

Björk GR, Huang B, Persson OP, Byström AS (2007) A conserved modified wobble nucleoside (mcm5s2U) in lysyl-tRNA is required for viability in yeast. RNA **13:** 1245-1255

Chan TF, Carvalho J, Riles L, Zheng XF (2000) A chemical genomics approach toward understanding the global functions of the target of rapamycin protein (TOR). Proc Natl Acad Sci U S A **97:** 13227-13232

Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol **133:** 462-469

Diet A, Brunner S, Ringli C (2004) The *enl* mutants enhance the *lrx1* root hair mutant phenotype of *Arabidopsis thaliana*. Plant Cell Physiol **45:** 734-741

Escobar-Restrepo JM, Huck N, Kessler S, Gagliardini V, Gheyselinck J, Yang WC, Grossniklaus U (2007) The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception. Science **317:** 656-660

Furukawa K, Mizushima N, Noda T, Ohsumi Y (2000) A protein conjugation system in yeast with homology to biosynthetic enzyme reaction of prokaryotes. J Biol Chem **275:** 7462-7465

Ghaemmaghami S, Huh WK, Bower K, Howson RW, Belle A, Dephoure N, O'Shea EK, Weissman JS (2003) Global analysis of protein expression in yeast. Nature **425:** 737-741

Gleave AP (1992) A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Mol Biol **20:** 1203-1207

Goehring AS, Rivers DM, Sprague GF (2003a) Attachment of the ubiquitin-related protein Urm1p to the antioxidant protein Ahp1p. Eukaryot Cell **2:** 930-936

Goehring AS, Rivers DM, Sprague GF (2003b) Urmylation: a ubiquitin-like pathway that functions during invasive growth and budding in yeast. Mol Biol Cell **14:** 4329-4341

Hochstrasser M (2009) Origin and function of ubiquitin-like proteins. Nature **458:** 422-429

Huang S, Bjornsti MA, Houghton PJ (2003) Rapamycins. Cancer Biol Ther **2:** 222-232

Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O'Shea EK (2003) Global analysis of protein localization in budding yeast. Nature **425:** 686-691

Kerscher O, Felberbaum R, Hochstrasser M (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins. *In* Annu Rev Cell Dev Biol **22:** 159-180

Kessler D (2006) Enzymatic activation of sulfur for incorporation into biomolecules in prokaryotes. FEMS Microbiol Rev **30:** 825-840

Krause K, Krupinska K (2009) Nuclear regulators with a second home in organelles. Trends Plant Sci **14:** 194-199

Leiber RM, John F, Verhertbruggen Y, Diet A, Knox JP, Ringli C (2010) The TOR pathway modulates the structure of cell walls in Arabidopsis. Plant Cell **22:** 1898-1908

Leidel S, Pedrioli PG, Bucher T, Brost R, Costanzo M, Schmidt A, Aebersold R, Boone C, Hofmann K, Peter M (2009) Ubiquitin-related modifier Urm1 acts as a sulphur carrier in thiolation of eukaryotic transfer RNA. Nature **458:** 228-232

Lian F-M, Yu J, Ma X-X, Yu X-J, Chen Y, Zhou C-Z (2012) Structural snapshots of yeast alkyl hydroperoxide reductase Ahp1 peroxiredoxin reveal a novel two-cysteine mechanism of electron transfer to eliminate reactive oxygen species. J Biol Chem **287:** 17077-17087

Minet M, Dufour ME, Lacroute F (1992) Cloning and sequencing of a human cDNA coding for dihydroorotate dehydrogenase by complementation of the corresponding yeast mutant. Gene **121:** 393-396

Miranda HV, Nembhard N, Su D, Hepowit N, Krause DJ, Pritz JR, Phillips C, Söll D, Maupin-Furlow JA (2011) E1- and ubiquitin-like proteins provide a direct link between protein conjugation and sulfur transfer in archaea. Proc Natl Acad Sci U S A **108:** 4417-4422

Noma A, Sakaguchi Y, Suzuki T (2009) Mechanistic characterization of the sulfur-relay system for eukaryotic 2-thiouridine biogenesis at tRNA wobble positions. Nucleic Acids Res **37:** 1335-1352

Park HJ, Kim WY, Park HC, Lee SY, Bohnert HJ, Yun DJ (2011) SUMO and SUMOylation in plants. Mol Cells **32:** 305-316

Petroski MD, Salvesen GS, Wolf DA (2011) Urm1 couples sulfur transfer to ubiquitin-like protein function in oxidative stress. Proc Natl Acad Sci U S A **108:** 1749-1750

Ringli C (2005) The role of extracellular LRR-extensin (LRX) proteins in cell wall formation. Plant Biosyst **139:** 32-35

Schlieker CD, Van der Veen AG, Damon JR, Spooner E, Ploegh HL (2008) A functional proteomics approach links the ubiquitin-related modifier Urm1 to a tRNA modification pathway. Proc Natl Acad Sci U S A **105:** 18255-18260

Stintzi A, Browse J (2000) The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci U S A **97:** 10625-10630

Teschner J, Lachmann N, Schulze J, Geisler M, Selbach K, Santamaria-Araujo J, Balk J, Mendel RR, Bittner F (2010) A novel role for Arabidopsis mitochondrial ABC transporter ATM3 in molybdenum cofactor biosynthesis. Plant Cell **22:** 468-480

Van der Veen AG, Schorpp K, Schlieker C, Buti L, Damon JR, Spooner E, Ploegh HL, Jentsch S (2011) Role of the ubiquitin-like protein Urm1 as a noncanonical lysine-directed protein modifier. Proc Natl Acad Sci U S A **108:** 1763-1770

Vierstra R (2012) The expanding universe of ubiquitin and ubiquitin-like modifiers. Plant Physiol doi:10.1104/pp.112.200667

Wang F, Liu M, Qiu R, Ji C (2011) The dual role of ubiquitin-like protein Urm1 as a protein modifier and sulfur carrier. Protein Cell **2:** 612-619

Xu J, Zhang J, Wang L, Zhou J, Huang H, Wu J, Zhong Y, Shi Y (2006) Solution structure of Urm1 and its implications for the origin of protein modifiers. Proc Natl Acad Sci U S A **103:** 11625-11630

Zhao Y, Dai X, Blackwell HE, Schreiber SL, Chory J (2003) SIR1, an upstream component in auxin signaling identified by chemical genetics. Science **301:** 1107-1110

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol **136:** 2621-2632

4.3 *ROL5* is connected to TOR signaling

4.3.1 ROL5 has two independent protein functions

It has been shown previously that *ROL5* is involved in the thiolation of specific tRNAs as well as in the process of TOR signaling. If the *ROL5* gene is mutated, the formation of modified tRNAs is no longer performed. Furthermore, mutation of *ROL5* leads to a rapamycin hypersensitivity phenotype (John et al., 2011). In yeast it has been shown that ROL5 is a functional and structural homolog to Ncs6p (Leiber et al., 2010*).* If *NCS6* is mutated (*Δncs6*) these mutants react hypersensitive to the TOR kinase-inhibiting drug rapamycin, and they are no longer able to perform the thiolation of tRNAs (Kozminski et al., 2003, Noma et al., 2009). To elucidate whether these functions represent two independent activities of ROL5, deletion constructs of *ROL5* were analyzed for their ability to complement for one or both of these *Δncs6* phenotypes. It is known that a full-length construct of *ROL5* is able to complement for both *Δncs6* phenotypes (Leiber et al., 2010). One N-terminal deletion of *ROL5* was constructed which is lacking the first 66 nucleotides, subsequently referred to as *Δ66_ROL5*, and transformed into *Δncs6* mutants. This complemented strain was then treated with 2.4 nM rapamycin. While *Δncs6* mutants complemented with a full-length construct of *ROL5* were able to grow comparable to wild type levels, strains complemented with *Δ66_ROL5* were inhibited in growth in the presence of rapamycin (Figure 3).

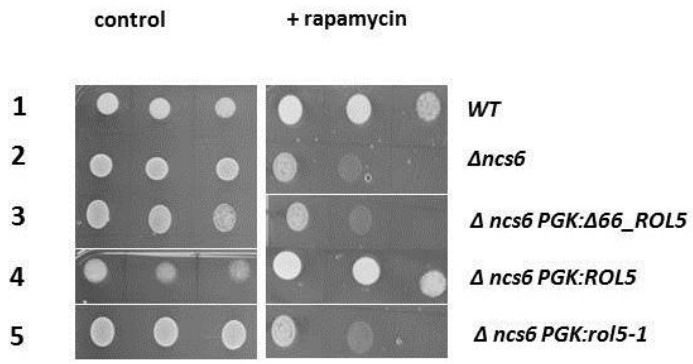

Figure 3 Yeast *Δncs6* mutants show a growth defect upon treatment with rapamycin. *Δncs6* mutants are hypersensitive to rapamycin (lanes 1+2). This effect is complemented with *ROL5* but not with *Δ66_ROL5* and *rol5-1* mutants (lanes 3+4+5). Under control conditions, all strains show comparable growth.

In a second step, *Δncs6 PGK*:*Δ66_ROL5* strains were tested for their ability to perform 2-thiouridine modifications of tRNAs. Bulk tRNA was extracted, and run on an APM (N-acryloylamino phenyl mercuric chloride) polyacrylamide gel. APM binds to thiolated tRNAs, and retards their migration in the gel. This produces a shift in band migration. In the wild type as well as in the *Δncs6 PGK*:*Δ66_ROL5*, in the *Δncs6 PGK*:*ROL5*, and in the *Δncs6 PGK*:*NCS6* strain, a shifted band indicated the accumulation of thiolated tRNAs (Figure 4). Hence, in yeast Δ66_ROL5 is able to perform 2-thiouridine modifications of tRNAs but is not able to complement for the rapaymcin hypersensitivity phenotype in *Δncs6* background. This suggests a dual function of ROL5.

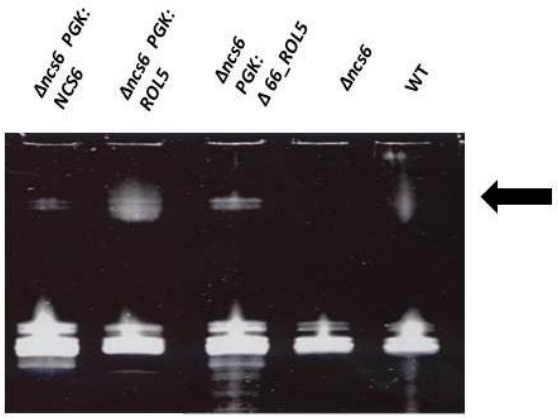

Figure 4 Complementation of the *Δncs6* tRNA thiolation phenotype. *Δncs6* mutants are lacking the ability to perform 2-thiouridine modifications of cytoplasmic tRNAs. This phenotype can be complemented by transformation of the *Δncs6* strain with full length *NCS6*, *ROL5* or an N-terminal deletion construct of *ROL5 (Δ 66_ROL5)*. Bulk tRNA was extracted from WT, *Δncs6,* and the complemented strains, respectively, and separated on a 8% Polyacrylamid Gel supplemented with 7M Urea+ [(N-acryloylamino) phenyl] mercuric chloride. A shift in band migration represents the thiolated tRNA species, indicated by an arrow.

Since the described dual function of ROL5 was only shown in yeast, further experiments were designed to check if ROL5 has the same activity in *Arabidopsis thaliana.* In Arabidopsis it has been shown that *rol5-1* is suppressing the phenotype caused by a mutation in the gene *LRX1.* The *lrx1* root hair phenotype is characterized by aberrant root hair formation, and bulbus like structures at the root hair basis that frequently burst (Baumberger et al., 2001). In *lrx1 rol5-1*double mutants a wild type-like appearance is restored (Leiber et al., 2010). To test if Δ66_ROL5 is still functional in *Arabidopsis thaliana*, *lrx1 rol5-1* double mutants were transformed with *Δ66_ROL5* under control of the native *ROL5* promoter. If Δ66_ROL5 was still functional, this is supposed to lead to reestablishment of the *lrx1* phenotype. However, homozygous complementation lines did not restore the *lrx1* phenotype but instead induced a short-root phenotype with primary roots that are shorter than in the *lrx1 rol5-1* double mutant (Figure 5).

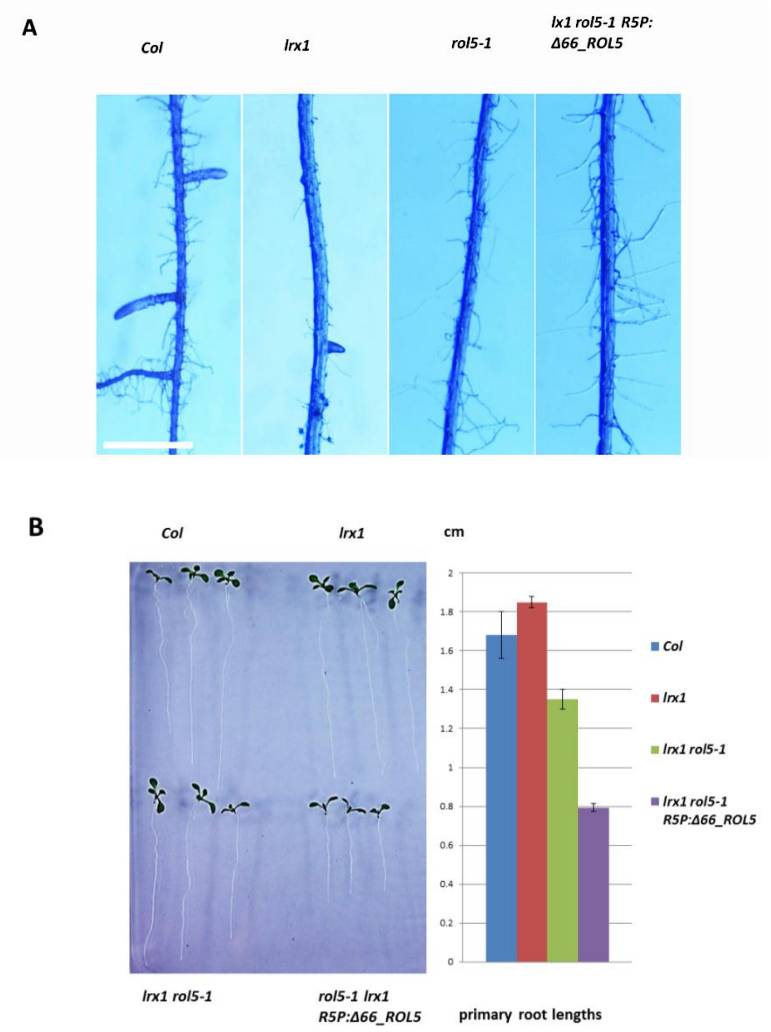

Figure 5 Phenotype of *lrx1 rol5-1 R5P: Δ66_ROL5* complementation lines. Complementation of a *lrx1 rol5-1* double mutant with *Δ66_ROL5* (*lrx1 rol5-1 R5P: Δ66_ROL5*) does not restore the *lrx1* phenotype **(A)** but results in a dominant negative effect leading to a primary root length shorter than *lrx1 rol5-1* **(B)**. Seedlings were grown vertically for seven days on half strength MS-Media. For measurement of primary root length n=20. Bar = 5 mm.in **(A)** and 3 mm in **(B)**.

Interestingly, this effect was not observed in *Col* wild-type plants expressing the same construct. To test if the observed phenotype is dependent on the *rol5-1* mutation only, *lrx1 rol5-1 R5P:Δ66_ROL5* plants were crossed with *Col* or *rol5-1*. In the F_1 generation only crosses with *rol5-1*, but not with *Col*, exhibited the dominant negative effect of *Δ66_ROL5*. This indicates that the observed root phenotype in *lrx1 rol5-1 R5P: Δ66_ROL5* is indeed dependent on the *rol5-1* mutation. To check for

the presence of 2-thiouridine modified tRNAs, bulk tRNA was extracted, and run on a polyacrylamid gel supplemented with APM. In the *lrx1 rol5-1* double mutant complemented with *R5P:Δ66_ROL5*, a retarded band indicated the presence of modified tRNAs even though at a reduced level (Figure 6).

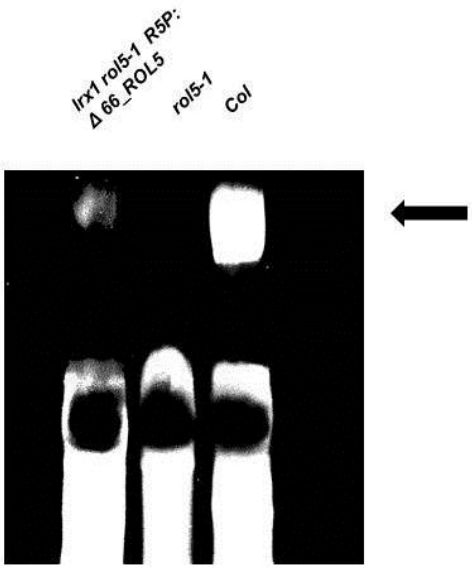

Figure 6 Δ 66_ROL5 allows thiolation of tRNAs. In *lrx1 rol5-1*mutants the ability to thiolate tRNAs is impaired. Transformation of *lrx1 rol5-1* mutants with either *NCS6* or a N-terminal deletion construct of *ROL5 (Δ 66_ROL5)* restores the ability to thiolate tRNAs. Bulk tRNA was extracted from WT, *Δncs6* and the complemented strain and separated on a 8% Polyacrylamid Gel supplemented with 7M Urea+ [(N-acryloylamino) phenyl] mercuric chloride. A shift in band migration represents the thiolated tRNA species, indicated by an arrow.

In summary it can be stated that the N-terminal deletion of *ROL5* (*Δ66_ROL5*) is functional regarding the formation of 2-thiouridine both in Arabidopsis and in yeast but is not able to restore the *lrx1* phenotype in the *lrx1 rol5-1* double mutant nor complements for the yeast *Δncs6* rapamycin hypersensitivity phenotype, suggesting a dual function of ROL5 in tRNA modification and TOR signaling.

4.3.2 The small GTPase ROP6 is interacting with ROL5

In yeast it has been shown that a double mutant of *NCS6* and *CDC42* is lethal (Kozminski et al., 2003). This demonstrates a genetic interaction of *NCS6,* and *CDC42* in yeast. Cdc42p is a small GTPase, and belongs to the Rho family. It is involved in the establishment of cell-polarity, and necessary for bud emergence (Perez et al., 2010). To elucidate if a homolog to Cdc42p also exists in Arabidopsis, a Blast search with the Cdc42p sequence against *Arabidopsis thaliana* proteins was performed, and revealed the closest homolog to be ROP6. The ROP6 protein consists of 198aa and belongs to the class of ROP (Rho of plants) GTPases. In *Arabidopsis thaliana* it has been shown that ROP6 is involved in the ordering of cortical microtubuli, and thus influences cell expansion (Fu et al., 2009). To identify a possible genetic interaction of *ROL5*, and *ROP6*, mutant lines were crossed to obtain a *rol5-1 rop6-1* double mutant. The *rop6-1* mutant is a T-DNA insertion mutant and likely to be a knockout-allele (Fu et al., 2009). When these lines were grown on half strength MS-Media the *rol5-1 rop6-1* double mutant showed a severe reduction in primary root growth in comparison to the wild type, *rop6-1*, and the *rol5-1* single mutant (Figure 7).

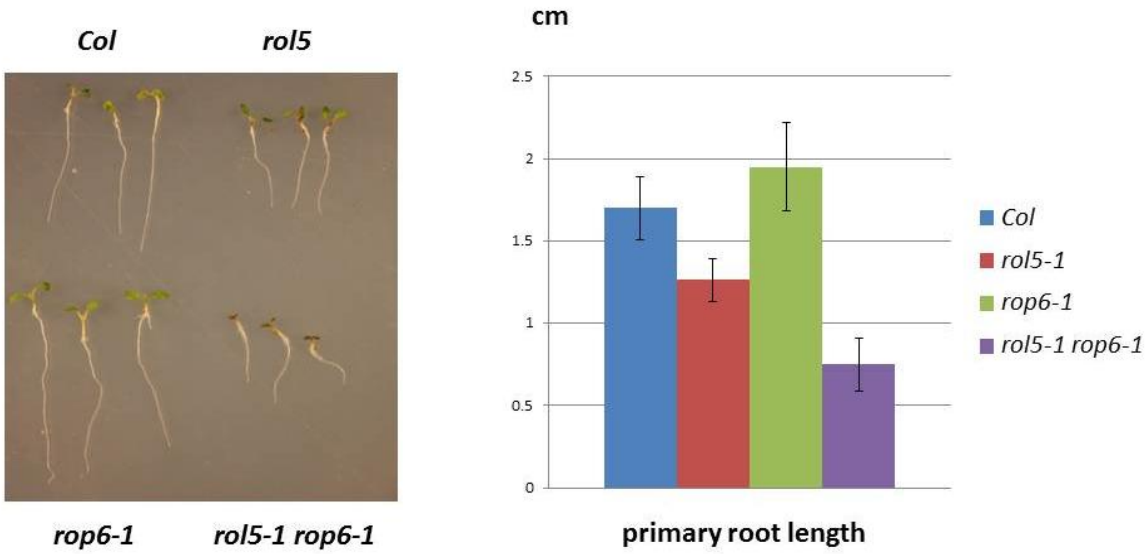

Figure 7 Genetic interaction of *ROL5* and *ROP6*.

Seedlings were grown vertically for seven days on half strength MS-media. The *rol5-1 rop6-1* double mutant seedlings show significantly shorter primary roots than the WT, *rol5-1* or *rop6-1* single mutants. Bar = 10 mm.

To check if there is also a physical interaction between ROL5, and ROP6, a Y2H experiment was performed. To this end, *ROP6* was cloned into *pGAD-HA*, and *ROL5* into *pLexA-N*. In this assay an interaction could be observed (Figure 8A). To confirm this result by an alternative experiment, an immuno-precipitation was done, using a C-terminal HA-tagged version of *ROL5* (*ROL5_HA*) cloned into *pFL61*, and a N-terminal HIS-tagged version of *ROP6* (*HIS_ROP6*) cloned into *pNEV-E*, two plasmids with constitutively active yeast promoters. A yeast strain containing both constructs was produced and subsequent immuno-precipitation with a Roche anti-HA matrix was performed. Again an interaction could be observed. A yeast strain expressing *HIS_ROP6* only was used as negative control (Figure 8B).

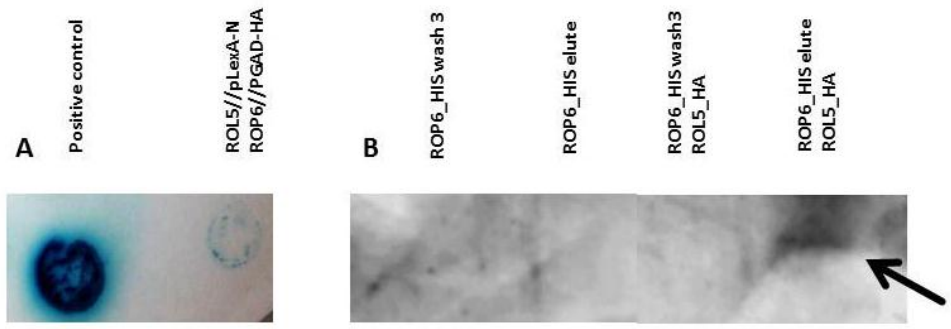

Figure 8 Physical interaction of ROL5, and ROP6. **(A)** In a Y2H assay ROL5 and ROP6 show a weak interaction signal. The blue color indicates the strength of interaction. **(B)** Yeast cells were produced containing both constructs *ROL5_HA*, and *HIS_ROP6*. In a second step ROL5 was immuno precipitated with an anti HA-matrix, and co-precipitation of ROP6 was tested with an anti-HIS antibody. While in the negative control (yeast cells transformed with *HIS_ROP6* only) no HIS_ROP6 protein is precipitated, the HIS_ROP6 band in the yeast strain containing both constructs , indicates an interaction between the two proteins.

Since our results suggest a physical interaction of ROP6 and ROL5, we wanted to further analyze whether the localization pattern of both proteins favors such an interaction *in vivo*. To this end, yeast cells were produced expressing N-terminal GFP fusions of *ROL5* (*GFP_ROL5*) or *ROP6* (*GFP_ROP6*). For ROP6, GFP fluorescence could then be observed mainly at the plasma membrane, but also in the cytoplasm (Figure 9A). For ROL5, GFP fluorescence was visible in the cytoplasm, and in distinct parts of the cytoplasm, possibly representing mitochondria, since a mitochondrial localization of ROL5 has been shown previously in Arabidopsis (Leiber et al, 2010) (Figure 9B). This fractional co-localization of ROP6 and ROL5 in the cytoplasm demonstrates that a physical interaction of both proteins is feasible.

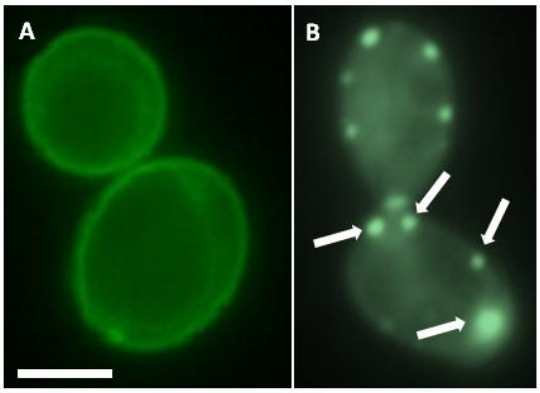

Figure 9 Localization of GFP_ROP6, and GFP_ROL5 fusion proteins in yeast. **(A)** An N-terminal GFP-fusion of ROP6 localizes to the membrane, and to the cytoplasm. **(B)** An N-terminal GFP-fusion of ROL5 localizes to the cytoplasm, and to distinct parts of the cytoplasm (arrows), potentially representing mitochondria. The mitochondrial localization of ROL5 has been shown previously (Leiber et al., 2010). Bar = 5 µm.

4.3.3 *ROP6* is interfering with TOR signaling

For the ROP6 (Rho of plant 6) interacting protein ROL5 it has been demonstrated previously that it interferes with TOR signaling (Leiber et al., 2010). This raises the question whether ROP6 is also influencing TOR. Recently, it could be shown in yeast that Rho1p, a member of Rho-family proteins, is complexing with the TOR complex 1 (TORC1), upon treatment with rapamycin or exposure to several stress conditions (Yan et al., 2012). In Arabidopsis TOR activity is, amongst others, linked to osmotic stress resistance. When *TOR*-silenced lines were exposed to osmotic stress, a significant reduction in primary root growth was observed (Deprost et al., 2007). To check for a putative genetic interaction of *ROL5*, *ROP6*, and *TOR*, mutants of *ROL5* (*rol5-1), ROP6 (rop6-1)*, and the respective double mutant were grown on half-strength MS-Media supplemented with 5% Mannitol. While the wild type, *rol5-1*, and *rop6-1* were germinating similarly, the *rol5-1 rop6-1* double mutant was severely affected (Figure 10).

This result shows that the *rol5-1 rop6-1* double mutant is less resistant to osmotic stress, likewise *TOR* silenced lines.

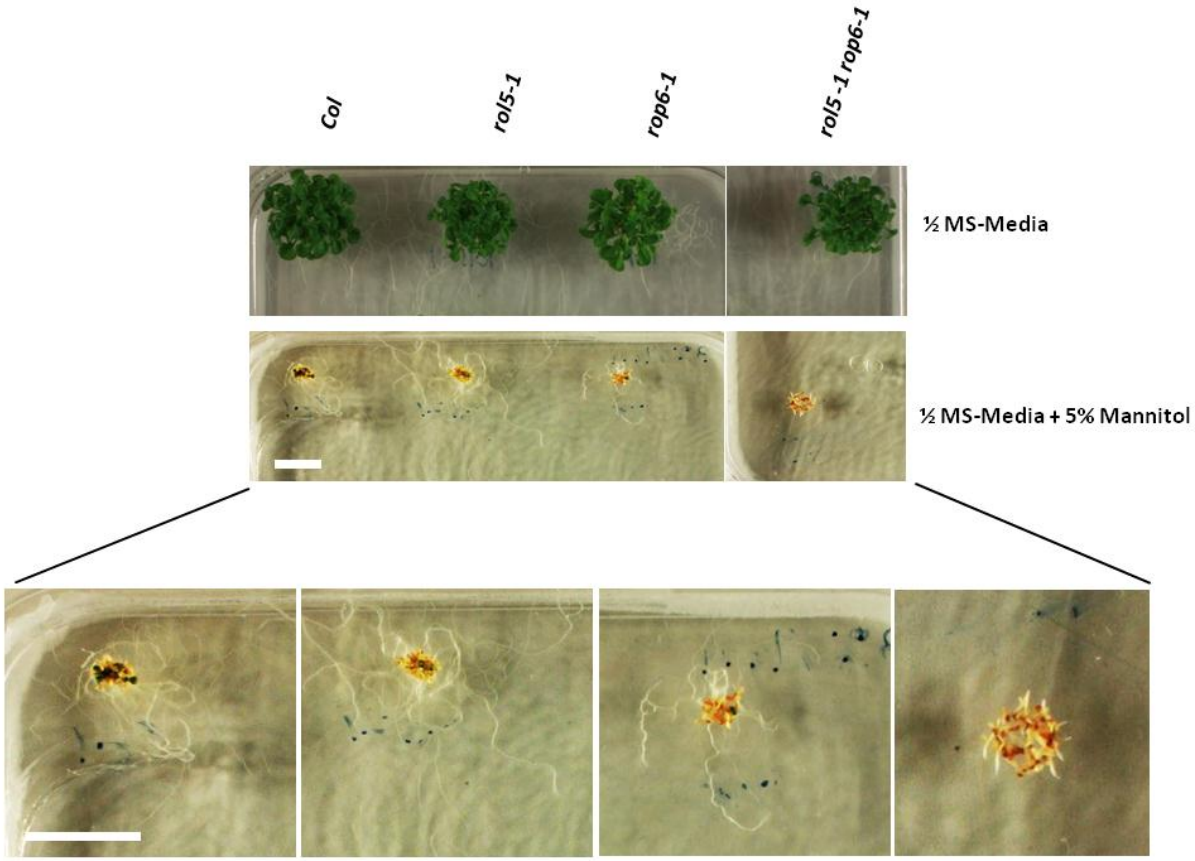

Figure 10 Osmotic-stress phenotype of the *rol5-1 rop6-1* double mutant. *rol5-1 rop6-1* double mutants show reduced germination compared to the wild type and the single mutants when grown on 5% Mannitol. All lines were grown horizontally on half-strength MS-media plates supplemented with 5% Mannitol for 21 days. Bar = 20 mm.

To get further evidence for ROP6 being involved in TOR signaling, *Col*, *rol5-1*, *rop6-1*, and, *rol5-1 rop6-1* plants were tested for their ability to cope with changes in nutrient availability. Nutrient abundance is a regulator of TOR activity in mammals as well as in plants (Menand et al., 2004; Wullschleger, 2006). When *rol5-1 rop6-1* double mutant seedlings were grown under sucrose depletion, a severe reduction in primary root length could be observed (Figure 11).

This gives further evidence for an involvement of *ROP6* in TOR signaling.

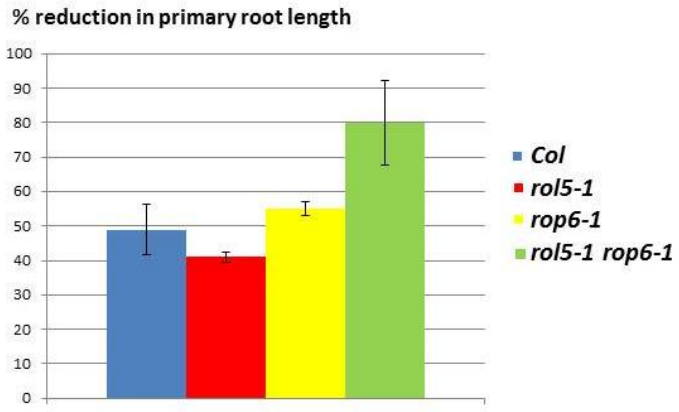

Figure 11 Nutrient sensing phenotype of the *rol5-1 rop6-1* double mutant. *rol5 rop6-1* double mutants show a stronger reduction in primary root length when grown on sucrose-depleted media than the wild type, *rol5-1,* or *rop6-1* mutants. The reduction was calculated by determining the average difference in root length of plants grown with or without sucrose.

4.3.4 Analysis of S6K1 activity in Arabidopsis

It has been shown in mammals that pp70S6K (S6K) is a target of phosphorylation through the TOR kinase which subsequently promotes protein translation (Magnuson et al., 2012). In Arabidopsis two S6K proteins are known. For S6k1, an interaction with the TOR associated protein (RAPTOR) has been shown which regulates the response to osmotic stress (Mahfouz et al., 2006). Furthermore, it was demonstrated that rapamycin-dependent inhibition of TOR leads to a lower phosphorylation status of S6K1 (Xiong, and Sheen, 2012). These experiments were done in protoplasts with overexpression of S6K1, which represents an artificial system. Therefore, we wanted to check whether also the phosphorylation level of natively expressed S6K1 protein is TOR-dependent. To this end, from *Arabidopsis thaliana* seedlings grown in liquid culture S6K was purified with an antibody raised against human S6K. Since S6K1 and S6K2 share high sequence homology, the antibody used is likely to detect both proteins (Mahfouz et al., 2006). The purified S6K proteins were then tested for their

phosphorylation status with an antibody recognizing the phosphorylation site of human S6K (Thr389). This corresponds to *Arabidopsis thaliana* S6k1 phosphorylation site Thr449 (Xiong, and Sheen, 2012). Both, *Co*l and *Col* 35S:*FKBP12* were treated with 5µg/ml rapamycin. In this experiment, no visible differences in S6K1 phosphorylation could be detected (Figure 12).

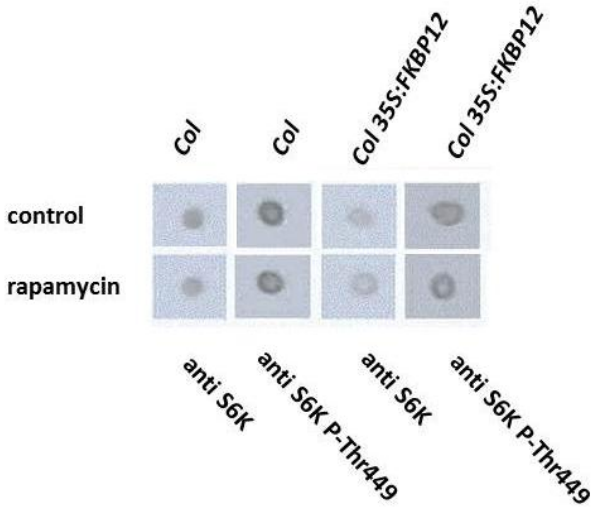

Figure 12 S6k Thr389-phosphorylation status of rapamycin treated *Col*, and *Col 35S:FKBP12*. Arabidopsis seedlings were grown in liquid culture supplemented with 5µg/ml rapamycin for 14 days. Native S6k protein was antibody purified, and equal amounts of S6k were determined by using an antibody recognizing the whole S6K molecule. The phosphorylation status was subsequently determined with an anti-P-Thr389 antibody. *Col 35S:FKBP12*, and *Col* show no reduction of S6K1 phosphorylation when treated with rapamycin.

When *rol5-1, rop6-1*, and *rol5-1 rop6-1* double mutants, which are thought to interfere with TOR signaling were tested for S6K1 phosphorylation, again no reduction in S6K1 activity could be detected in comparison with the wild type (Figure 13). This means that our approach is not suitable or not sensitive enough for detecting subtle differences in native S6K1 phosphorylation in Arabidopsis.

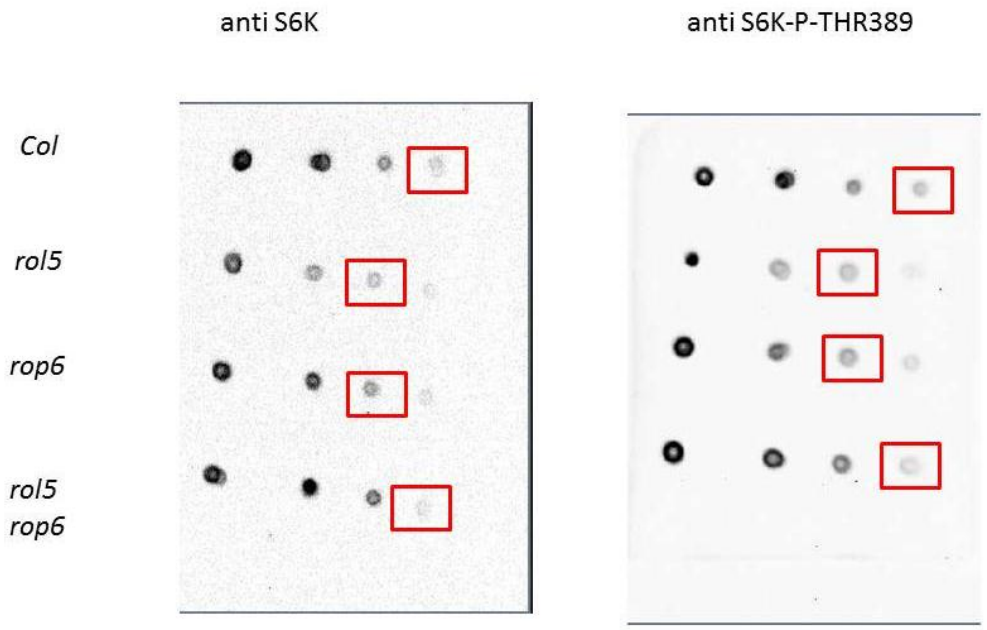

Figure 13 S6K THR389 phosphorylation status of *Arabidopsis thaliana* mutants. The left panel shows the amount of S6K protein in 4 decreasing dilutions. The right panel shows the phosphorylation level of S6K at phosphorylation site THR389. Red squares indicate comparable dilution levels. No difference in between the mutants and *Col* is detectable.

4.4 AtNCS2 is important for primary root development

In yeast Ncs2p is an essential component of tRNA thiolation and has been shown to bind to Ncs6p (Leidel et al., 2009). The Arabidopsis homolog is encoded by the locus At4g35910, subsequently referred to as *AtNCS2*. An alignment of *AtNCS2,* and yeast *NCS2* reveals an identity of 21% (Figure 14).

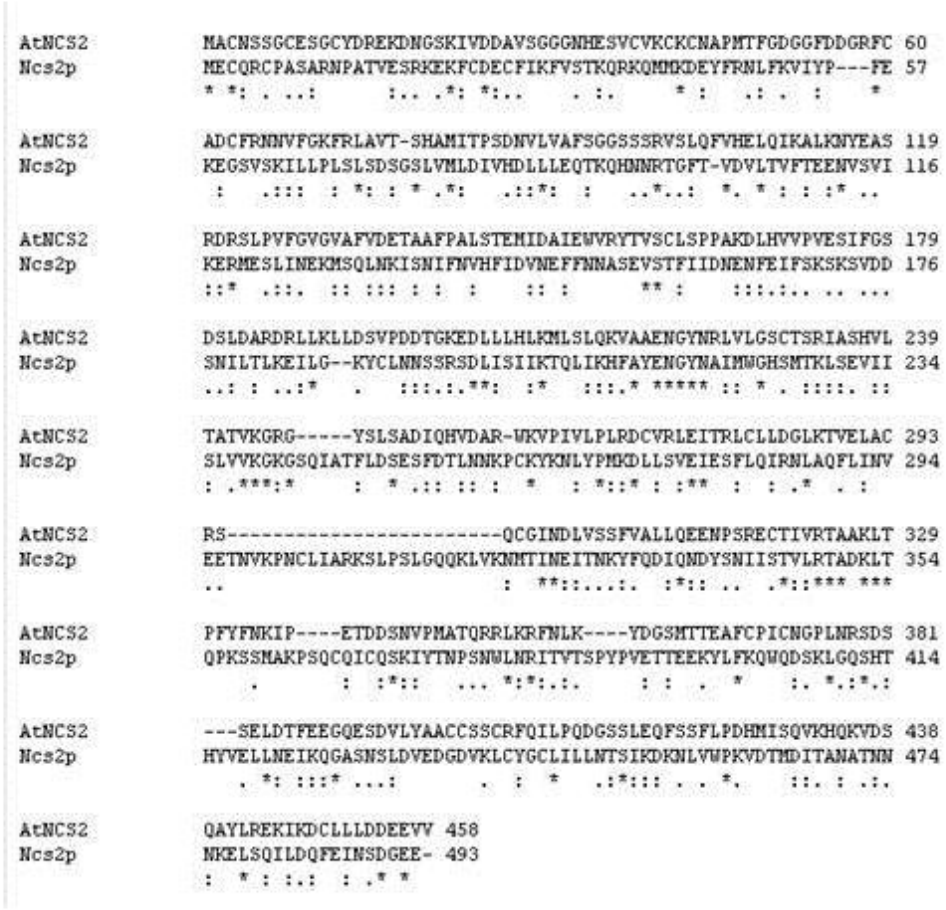

Figure 14 Alignment of AtNCS2, and Ncs2p. Both proteins share an identity of 21%. To check whether *AtNCS2* is actually expressed, the expression pattern was analyzed in Arabidopsis. To this end, an *AtNCS2* promoter-*GUS* fusion was transformed into Arabidopsis wild type plants and GUS activity was visualized by Xgluc staining. In all lines, GUS activity was strongly observed in all tissues of 7 days old seedlings (Figure 15). This indicates an ubiquitous expression of *AtNCS2*.

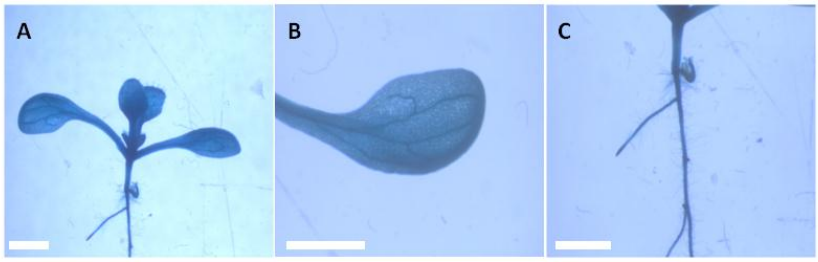

Figure 15 Expression pattern of *AtNCS2.* Arabidopsis WT plants were transformed with an *AtNCS2*-promoter-*GUS* fusion. GUS construct activity was then assessed at the seedling stage using a light microscope. The *AtNCS2* promoter is active in all tissues. Bars = 4 mm.

In yeast, Ncs2p is interacting with the ROL5 homolog Ncs6p. This interaction is essential for the formation of thiolated tRNAs (Leidel et al, 2009). An interaction of ROL5 with AtNCS2 would indicate a functional conservation of Ncs2p, and AtNCS2. To elucidate this, an Y2H with Ncs2p and AtNCS2 was performed. To this end, *ROL5* was cloned into *pGAD-HA* and *AtNCS2* into *pLexA-N*. In this assay, a strong interaction of both proteins could be detected (Figure 16). This gives first evidence for a conservation of the function of Arabidopsis and yeast.

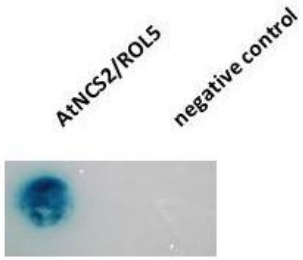

Figure 16 AtNCS2 is interacting with ROL5. A Y2H assay with *ROL5* cloned into *pGAD-HA* and *AtNCS2* cloned into *pLexA-N* with subsequent filter lift-off assay was performed. The blue staining indicates an interaction of both proteins.

To get further evidence whether AtNCS2 is functional similar to Ncs2p, yeast *Δncs2* mutants were complemented with a cDNA construct of *AtNCS2* under control of a constitutive active yeast promoter (*Δncs2 PGK:AtNCS2)*. Yeast *Δncs2* mutants have no detectable production of thiolated tRNAs. Being functionally similar, the complementation with AtNCS2 should therefore restore the presence of thiolated tRNAs. To check for the presence of thiolated tRNAs, bulk tRNA was extracted from *Δncs2 PGK:*AtNCS2, *Δncs2*, and the wild type. Subsequently, purified tRNA was run on an acrylamide gel supplemented with N-acryloylamino phenyl mercuric chloride (APM). APM binds to thiolated tRNAs, and retards their migration in the gel. This produces a band-shift. In this assay, the wild type was shown to be able to thiolate tRNAs whereas *Δncs2* mutants, and *Δncs2 PGK:AtNCS2* had no detectable levels of thiolated tRNAs (Figure17). The expression of *AtNCS2* was confirmed by RT-PCR (data not shown). This means that AtNCS2 is not functional in yeast.

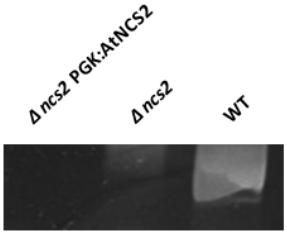

Figure 17 Arabidopsis and yeast *NCS2* are not exchangeable. *Δncs2* mutants are lacking the ability to thiolate tRNAs. A *Δncs2* strain expressing *AtNCS2* (*Δncs2 PGK:AtNCS2*) was analysed for the presence of thiolated tRNAs. Bulk tRNA was extracted from WT, *Δncs6,* and the complemented strains, respectively, and separated on an 8% Polyacrylamid Gel supplemented with 7M Urea+ [(N-acryloylamino) phenyl] mercuric chloride. A shift in band migration indicates the present of modified tRNAs. Only shifted bands are shown. Yeast *Δncs2 PGK:AtNCS2* does not accumulate thiolated tRNAs. Therefore, Arabidopsis *AtNCS2* is not complementing *Δncs2*.

Yeast represents a heterologous system. So far, no function of *AtNCS2* is known in Arabidopsis. To investigate the biological relevance of *AtNCS2* in plant development, two T-DNA insertion lines were

used for analysis. Salk-30197 corresponds to allele *ncs2*-1 and GK-686B10-022973 corresponds to allele *ncs2-2*. The insertion sites were defined and for *ncs2-2* found to be situated at nucleotide position 879 in the third exon. Therefore *ncs2-2* is thought to be a knockout-allele. In contrast, *ncs2-1* was found to be situated in the terminator. Consequently, only allele *ncs2-2* was used for subsequent analysis (Figure 18).

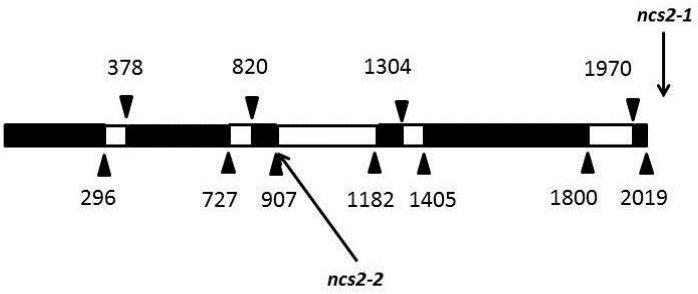

Figure 18 Genomic structure of *AtNCS2*. Black boxes represent exons, and white boxes introns. The insertion in allele *Δncs2-1 (*Salk-30197) is located in the terminator and the insertion in *Δncs2-2* (GK-686B10-022973) is located at nucleotide 879 in the third exon. The two insertions are indicated by arrows.

The phenotype of the *Δncs2-2* mutant is characterized by a severe defect in primary root growth. Primary roots of *Δncs2-2* are significantly shorter than wild-type roots. This indicates that *AtNCS2* is important for plant growth (Figure 19).

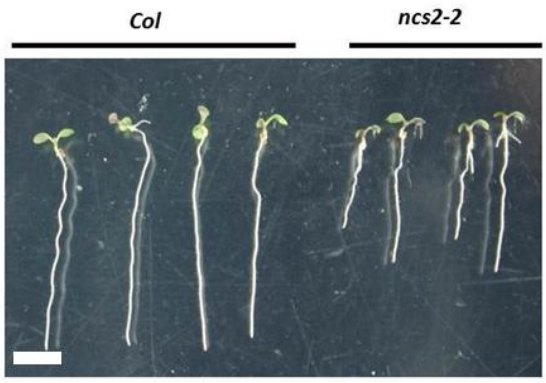

Figure 19 *ncs2-2* exhibits a severe reduction in primary root lengths. Plants were grown vertically on half-strength MS-media for seven days. Bar = 5 mm.

4.5 *HROL5* is a pseudogene

ROL5 has been shown to function in the process of thiolation modification of tRNAs and also interferes with TOR signaling (Leiber et al., 2010). In Arabidopsis one homologous gene (At1G76170) is present which is highly homologous to *ROL5* with 72% identity. This gene is referred to as *HROL5* (homolog of *ROL5*). To elucidate whether *HROL5* is expressed, a promoter-*GUS* fusion was analysed. To this end, a *HROL5* promoter-*GUS* construct was transformed into wild-type Arabidopsis and GUS activity was visualized by X-Gluc staining. GUS activity was detected in tissues of seedlings but especially in the veines and trichomes of rosette leaves (Figure 20).

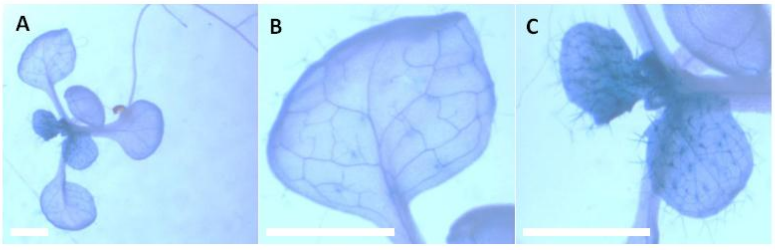

Figure 20 Expression pattern of the *HROL5.* GUS activity of *HROL5* promoter-*GUS* lines reveals the promoter activity to be distributed all over the whole seedling **(A),** but especially in the veines of rosette leaves **(B)**, and in the trichomes of rosette leaves **(C).** Bar = 5 mm.

To get an insight into the function of *HROL5* in plant development, a T-DNA insertion mutant was analysed. The T-DNA insertion is located in the second exon of the *HROL5* gene and is considered to result in a knockout-allele. This allele is subsequently referred to as *Δhrol5.* The *Δhrol5* mutant did not exhibit a phenotype. This raised the question whether the annotated *HROL5* sequence is correct. To elucidate this, the 5' end of the *HROL5* mRNA was determined by 5' RACE. This revealed that *HROL5* has a stop codon in frame at position 66. This position was formerly annotated to be part of the intronic region. This means that the annotated intron is wrong and an unusual splice site with the 5'-end of the intron (AGGTAAA…) is used. The vast majority of introns in Arabidopsis use the canonical GT-AG consensus sequence for the 5' and 3' end of the intron, respectively (Wu et al., 2012). This explains why prediction of the correct processed mRNA failed. In summary, *HROL5* is considered to be a pseudogene (Figure 21).

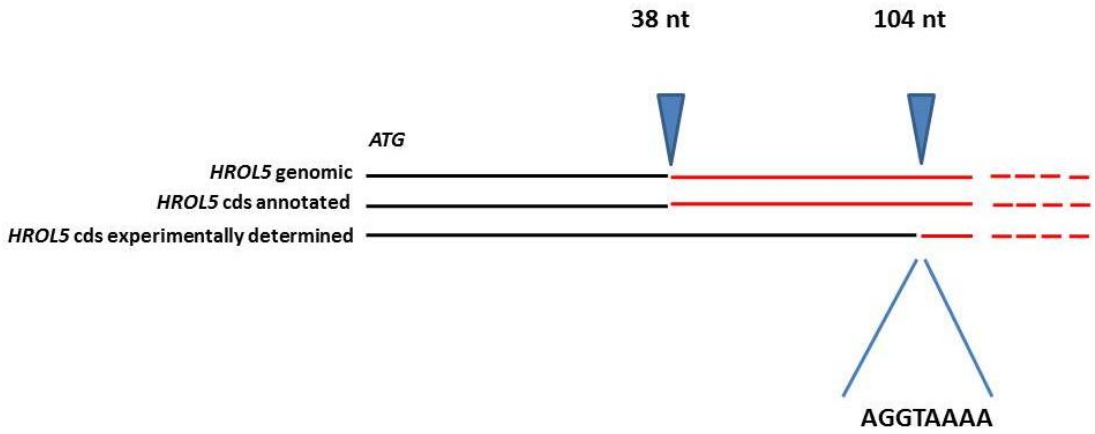

Figure 21 *HROL5* is a pseudogene. Alignment of the annotated coding sequence, the genomic sequence, and the experimentally determined cds of *HROL5* exhibits that the first stop codon is wrong annotated. Coding sequences are indicated by black lines and intronic regions by red lines. The determined splice site at the 5'-end of the intron (AGGTAAAA) is unusual. The vast majority of introns in Arabidopsis use the canonical GT-AG consensus sequence (Wu et al., 2012). This explains why prediction of the correct processed mRNA failed.

4.6 Analysis of *TOR* TILLING lines

It was demonstrated by Leiber et al. (2010) that the potent TOR inhibiting drug rapamycin is able to suppress the root hair phenotype of *lrx1* mutants. In a second, independent experiment we wanted to confirm the inhibition of TOR as specific mechanism of *lrx1* suppression. To this end, lines with altered TOR activity had to be identified for crossing with *lrx1*. A suppression of the *lrx1* phenotype in one of these mutants would demonstrate that TOR attenuation is responsible for *lrx1* suppression. A screen for *tor* TILLINGS mutants was performed and 11 alleles were identified (Table 1).

TILLING line	type of mutation	ABRC Stock
tor71	Splice Junction	CS93571
tor01	Non-coding	CS95001
tor17	H196=H196	CS93917
tor32	P197L	CS95232
tor83	V245I	CS96083
tor35	P294=P294	CS94835
tor63	P312L	CS94963
tor40	G344E	CS93540
tor97	A381V	CS93697
tor81	S399F	CS93681
tor66	Non-coding	CS93566

Table 1 Overview of *tor* TILLING alleles. Eleven *tor* TILLING alleles were identified. Six alleles are missense mutations leading to an amino acid exchange.

Only missense alleles leading to an amino acid exchange were used for further analysis. Subsequently, the *tor* missense alleles were screened for changes in primary root development, being an indicator for TOR activity. In addition, *tor* overexpression and *RNAi* lines were also screened for changes in primary root development. The overexpression and *RNAi* lines were obtained from

Christian Meyer, INRA Versailles. Three *tor* TILLING alleles showed a significant faster primary root development and one *RNAi* line exhibited a significant slower primary root development (Figure 22).

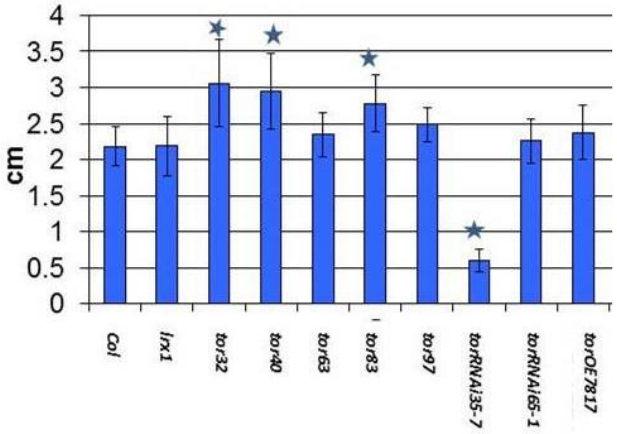

Figure 22 Primary root lengths of *tor* tilling, *tor RNAi*, and *TOR* overexpression lines. The stars indicate lines with altered root length (significance level with α=0.05). Plants were grown vertically for 7 days.

All lines were subsequently crossed with *lrx1*. The line *tor RNAi65-1 lrx1* showed a suppression of the *lrx1* phenotype (Figure 23). This suggests that indeed alteration of TOR activity is responsible for *lrx1* suppression. Intriguingly, *tor RNAi65-1* exhibited no severe change in primary root growth. Likewise, none of the TILLING lines showed a negative effect on root development. This indicates that all TILLING alleles and *tor RNAi65-1* had no clear negative effect on TOR-related cell growth.

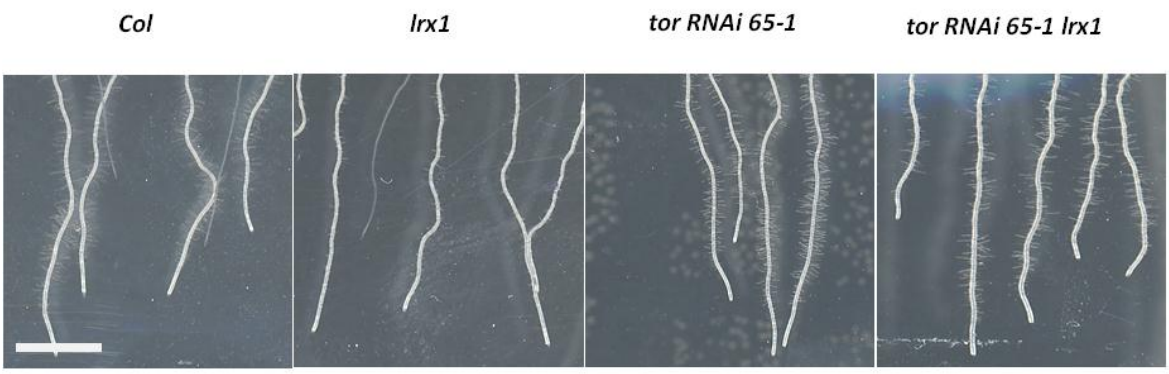

Figure 23 Crossing of *lrx1* with *tor RNAi 65-1* results in suppression of the *lrx1* phenotype. Plants were grown vertically for 7 days. Bar = 5 mm.

5 Discussion

To this day not much is known about how plant cells orchestrate the necessary adaptations in cell wall composition during adaptational responses and growth. The suppression of the *lrx1* root hair phenotype by *rol5-1* suggests an involvement of ROL5 in cell wall formation. Though, the underlying mechanism is not clear yet. On the basis of data obtained from yeast and humans, ROL5 could be experimentally linked to the TOR signaling pathway (Leiber et al., 2010). This raised the question whether TOR, as a central controller of growth processes is able to regulate not only growth, but also initiates essential cell wall adaptations. Due to the possibility to chemically suppress *lrx1* with rapamycin, evidence accumulated that TOR is indeed affecting the composition of the plant cell wall (Leiber et al., 2010).

5.1 ROL5 performs dual functions

Apart from TOR signaling, ROL5 could also be linked to the process of tRNA thiolation (Leiber et al., 2010). Therefore, a deeper analysis of ROL5 function shifted into focus. The thiolation of tRNAs is an important process for the accuracy of codon usage, and for translational efficiency (Rogers et al., 1995). The defect in tRNA thiolation of the *rol5-1* mutant could very well affect the expression of cell wall biosynthesis genes which in turn could explain the suppression of *lrx1* without involvement of TOR. TOR itself is also known to affect the expression of genes required for cell wall biosynthesis in fungi (Fuchs et al., 2009). Therefore, it can be speculated whether the observed suppression of *lrx1* is induced by alteration of TOR activity due to the *rol5-1* mutation or by a changed expression profile due to the lack of thiolated tRNAs in the *rol5-1* mutant. Previously performed microarray experiments, however, did not show changes in gene expression in *rol5-1* seedlings, making the latter hypothesis unlikely.

The fact that *Δ66_ROL5* allows the thiolation of tRNAs in *Δncs6* but does not complement the hypersensitivity to rapamycin gives evidence that ROL5 has two independent protein functions in the tRNA thiolation and in TOR signaling. This corroborates the theory that the suppression of *lrx1* could be induced by altered TOR activity. Since yeast represents a heterologous system, the function of *Δ66_ROL5* under control of the ROL5 promoter (R5P) was also tested in Arabidopsis *rol5-1 lrx1*. In *rol5-1 lrx1* background *Δ66_ROL5* allowed the thiolation of tRNAs but did not restore the *lrx1* phenotype, again suggesting a separation of tRNA thiolation and the suppression of *lrx1*. In addition, the *lrx1 rol5-1* R5P:*Δ66_ROL5* plants exhibit a dominant negative primary root phenotype. This effect can possibly be explained by an inhibitory function of *Δ66_ROL5* in Arabidopsis TOR signaling which cannot be observed in yeast (data not shown). Considering that no experimentally determined domain structure of ROL5 or Ncs6p is available, *Δ66_ROL5* could very well still contain parts of the domain responsible for interfering with Arabidopsis TOR. Ncs6p is very similar to ROL5 but exhibits weaker homology in the N-terminal region (Leiber et al., 2010). This might explain why *Δ66_ROL5*

expression in yeast does not have the same effect as in Arabidopsis. Therefore, it will be very interesting to elucidate in future whether a C-terminal deletion of *ROL5* or *NCS6* allows complementation of *Δncs6* rapamycin hypersensitivity but not tRNA thiolation. Furthermore, it will be interesting to test N- and C-terminal deletions of *NCS6* for their function in *lrx1 rol5-1* background. This would help identifying a functional domain structure of ROL5 and Ncs6p and differences in function of these proteins. Together, there is good evidence for *lrx1* suppression functioning through TOR activity alteration. In contrast, the process of tRNA thiolation seems to represent an independent, second function of ROL5.

5.2 *lrx1* is suppressed by alteration of TOR activity

To get a better understanding of TOR-related *lrx1* suppression, *tor* TILLING lines with missense mutations in the kinase domain of TOR were used for crossing with *lrx1*. Such alleles with different degrees of loss of function are useful because a *tor* knockout is lethal (Menand et al., 2002; Kurowska et al., 2011). In addition, *TOR RNAi* and *TOR* overexpression lines (Deprost et al., 2007) were analysed. TILLING takes advantage of classical EMS mutagenesis, providing a high frequency of point mutations randomly distributed over the genome with one mutation every 200-500 kb (Kurowska et al., 20011). This made it very promising to identify several *TOR* mutant alleles. In the end, we obtained 11 TOR alleles whereof 6 led to an amino acid exchange and one allele with a mutated splice junction. Three of the TILLING lines with amino acid exchange exhibited longer roots than *Col*. The other TILLING lines did not show a visible phenotype. An increase in primary root growth is reminiscent of the *TOR* overexpression phenotype (Deprost et al., 2007). This indicates that these three lines are representing autoactive versions of TOR with increased TOR activity. Alternatively, the binding site of a repressor might be affected. But to ensure that the observed effect on root growth is indeed TOR-dependent it will be necessary to complement these lines with the wild-type *TOR*. In addition, it will be essential to transform *Col* with the mutated versions of *TOR* to identify possible

semi-dominant effects. Intriguingly, only one *RNAi* line showed the described decrease in primary root growth, while further *RNAi* and overexpression lines had no visible phenotype. This is in contrast to the observation made by Deprost et al. (2007), but might be explained by silencing of the *RNAi* and overexpression constructs. All lines were subsequently crossed with *lrx1*. One *RNAi* line (*65-1*) showed a suppression of *lrx1* suggesting that reduced TOR activity leads to suppression of *lrx1*. Interestingly, apart from suppressing *lrx1*, this *RNAi* line had no visible mutant phenotype. Suppression of *lrx1* by an *RNAi* line that seems to be ineffective in silencing is somewhat puzzling. It has been shown in yeast that the rate of cell growth is dependent on TORC1 activity, while TORC2 is regulating cytoskeletal dynamics (Wullschleger et al., 2006). Therefore, suppression of *lrx1* might very well be established by action of TORC2 without being dependent on changes in the rate of cell growth. This implies that not a complete silencing of TOR takes place allowing TORC1 but not TORC2 to assemble and function. In contrast, *lrx1* is also suppressed by rapamycin (Leiber et al., 2010) which has been shown to only affect TORC1 in yeast and humans (Wullschleger et al., 2006). In Arabidopsis, the existence and function of TORC2 has not been described yet. Therefore, it is possible that Arabidopsis TORC2 unlike yeast and humans might be rapamycin sensitive and suppression of *lrx1* is induced by reduced TORC2 activity. This hypothesis, however, is very speculative and requires further investigation. In summary it can be said that *lrx1* suppression is very likely to be caused by altered TOR activity. Furthermore, rather changes in cytoskeletal dynamics than in cell growth might be responsible for *lrx1* suppression. But the exact mechanism still remains to be elucidated.

5.3 *ROP6* is involved TOR signaling

To obtain a better understanding of ROL5 function the knowledge of the yeast interactome (Breitkreutz et al., 2003) was used to identify interactors of Ncs6p since the Arabidopsis homologs consequently represent potential interactors of ROL5. In yeast, Cla4p was genetically linked to Ncs6p and is known to be an effector protein of the small GTPase Cdc42p (Kozminski et al., 2003; Richman

et al., 2004). Cdc42p, in turn is genetically linked to Ncs6p (Kozminski et al., 2003). Small GTPases have been shown to influence and control organization of the cytoskeleton, and vesicular trafficking in animals, fungi, and plants (Mucha et al., 2010). Cdc42p is a central mediator of polar growth, actin rearrangements, and cell cycle progression. Intriguingly, Cdc42p has been directly linked to TOR signaling (Wang et al., 2009). Such a link has also been demonstrated for the small GTPases Rho1 and Rhb1 (Fuchs et al., 2009; Tsao et al., 2009). Furthermore, TOR dependent regulation of small GTPase Rho1 has been demonstrated to be important for CWI sensing (Fuchs et al., 2009). This made it very promising that also in Arabidopsis, TOR activity and cell wall adaptations are linked via small GTPases. The Arabidopsis *CDC42* homolog *ROP6* exhibits only a subtle pavement cell shape phenotype (Fu et al., 2009). But a *rol5-1 rop6-1* double mutant shows a severe primary root growth phenotype. Considering that ROL5 is known to be involved in cell wall formation and TOR signaling, this corroborates the hypothesis that the small GTPase ROP6 is indeed involved TOR signaling in Arabidopsis. Furthermore, this implies a good conservation of this network in yeast and Arabidopsis. Unfortunately, no clear Arabidopsis homolog to *CLA4* could be identified to test for interaction with *ROL5*. Instead, ROL5 has been shown to physically interact with ROP6, while in yeast no such interaction between Cdc42p and Ncs6p has been shown. This might suggests that Cla4p got lost in the evolution of this pathway in Arabidopsis and was substituted by direct interaction of ROL5 and ROP6. Cla4p is a member of the p21-activated kinases (PAK) family of serine/threonine kinases. During polarized cell growth, Cla4p has been shown to be directed to the plasma membrane and is activated by Cdc42p (Lin et al., 2009). ROP6, like Cdc42p and Cla4p, has been shown to be important for polarized cell growth (Fu et al., 2009). Unfortunately, no kinase domain is present in ROL5 which is contradictory to the hypothesis that ROL5-ROP6 direct interaction makes a Cla4p homolog obsolete in Arabidopsis. Alternatively, it can also not be excluded that the Cla4p kinase is substituted in Arabidopsis by a different kinase not homologous to Cla4p. In Arabidopsis, the localization of ROP6 at the membrane and in the cytoplasm very well allows such an interaction since ROL5 has been shown to be localizing to the mitochondria (Leiber et al., 2010) as well as to the cytoplasm (chapter

2.3). For yeast Ncs6p, a mitochondrial localization has been proposed (Huh et al., 2003) while Cdc42p has been shown to exist in a soluble and a membrane-bound state (Ziman et al., 1993; Howson et al., 2005). Taking into account that Ncs6p is also involved in the thiolation of cytoplasmatic tRNAs, an additional localization in the cytoplasm is very likely (Leidel et al., 2009), but this remains to be elucidated. Unlike for yeast, a connection between small GTPases and TOR signaling has so far not been shown in Arabidopsis. This made us analyze further the connection between TOR and ROP6. In Arabidopsis osmotic stress resistance has been shown to be linked to TOR activity (Deprost et al., 2007). For this reason, it was very interesting to observe a strong germination phenotype of *rol5-1 rop6-1* mutants when grown on media containing 5% Mannitol, suggesting a link between TOR, ROL5, and ROP6. To dissect this link further, the ability of *rol5-1 rop6-1* to grow on sucrose-depleted media was tested. TOR is known to be a sensor for nutrient availability (Wullschleger, 2006). Intriguingly, the double mutant again exhibited a growth phenotype not seen in wild type or the single mutants, creating further evidence for a synergistic involvement of ROP6 and ROL5 in TOR signaling. Together, there is good evidence for the small GTPase ROP6 representing the link between ROL5, TOR signaling, and adaptations of the cell wall structure. This is corroborated by the fact that such a link has been described before, in yeast (Fuchs et al., 2009; Tsao et al., 2009; Wang et al., 2009). In Arabidopsis, it has been shown that changes in TOR activity lead to alterations in the cell wall composition (Leiber et al., 2010). Therefore, it will be interesting to see whether the *rop6-1* mutant also exhibits an aberrant cell wall structure. Nevertheless, it remains to be elucidated how TOR, ROL5, and ROP6 manage to orchestrate cell growth in accordance with the necessary changes in the cell wall structure.

5.4 Is TOR activity influenced by ROL5 and ROP6?

Our results suggest an involvement of ROL5 and ROP6 in TOR signaling. This is based on data obtained in yeast and a genetic approach in Arabidopsis. Consequently, it was interesting to analyze

whether TOR activity is indeed altered by action of ROL5 and ROP6. In mammals and in Arabidopsis, it has been shown that phosphorylation of kinase S6K is altered by TOR activity (Magnuson et al., 2012; Xiong, and Sheen, 2012). In Arabidopsis, 2 *S6K* homologs are known (*S6K1*, and *S6K2*), but only S6K1 phosphorylation at THR389 is influenced by TOR, which was demonstrated in protoplasts overexpressing *S6K1* (Xiong, and Sheen, 2012). Therefore, S6K1 phosphorylation is an ideal parameter for measuring TOR activity. Surprisingly, neither *rol5-1*, nor *rop6-1*, nor the double mutant exhibited an altered S6K phosphorylation status. Intriguingly, also our control (*Col 35S:FKBP12* treated with rapamycin) exhibited no change in S6K1 phosphorylation. This is remarkable since altered S6K1 phosphorylation upon rapamycin treatment has been reported before in Arabidopsis protoplasts overexpressing S6K1 (Xiong and Sheen, 2012). Our experiments were conducted without overexpression of S6K1 and thus relied on the endogenous S6K. Furthermore, the used human S6K THR389 antibody is also known to bind to S6K2 THR389 (Xiong and Sheen, 2012). Therefore, an alteration of S6K1 phosphorylation might be masked by native S6K2 phosphorylation. In this case, detection of changed S6K1 phosphorylation level might have to be done by overexpression of *S6K1*. In future, it would be interesting to elucidate whether it is possible to detect alterations of S6K1 phosphorylation in Arabidopsis *S6K2 RNAi* lines. This would indicate that S6K1 phosphorylation is indeed masked by S6K2 activity. Furthermore, our experiments will have to be repeated in protoplasts with overexpression of S6K1, as done by Xiong and Sheen (2012) since subtle changes in phosphorylation might be better visible. In mammals and yeast, only TORC1 is rapamycin sensitive and influences S6K1 phosphorylation, while TORC2 is interfering with the cytoskeleton (Wullschleger et al., 2006). ROP6 is also known to interact with the cellular cytoskeleton (Fu et al., 2009). Therefore, our results could indicate that ROL5 and ROP6 are only interfering with TORC2. As mentioned earlier, this is puzzling since rapamycin treatment of *lrx1* is mimicking *rol5-1* and rapamycin has been shown to only inhibit TORC1 in mammals and yeast. Nevertheless, to date TORC2 and its possible function in Arabidopsis has not been described. Therefore, in Arabidopsis a rapamycin sensitive TORC2 might exist without having an influence on S6K phosphorylation level. In

future, it will be very important to screen *rol5-1*, *rop6-1*, and *rol5-1 rop6-1* for possible defects in the cytoskeleton. Combined with the finding that *ROL5* and *ROP6* are genetically linked to TOR, this would create evidence for the existence of TORC2 in Arabidopsis.

5.5 Conservation of the tRNA thiolation network in Arabidopsis

Excessive work was done to elucidate the tRNA thiolation pathway in yeast and humans (Goehring et al., 2003a;b; Dewez et al., 2008; Schlieker et al., 2008; Leidel et al., 2009). In contrast, not much was known about a possible conservation in Arabidopsis. Therefore, we checked Arabidopsis homologs for their possible function in tRNA thiolation. AtURM1 and AtURM2 were identified to be structural and functional homologs to yeast Urm1p, suggesting a conservation of the tRNA thiolation pathway, also in Arabidopsis.

In yeast, also Ncs2p is essential for tRNA thiolation which requires an interaction of Ncs2p with Ncs6p (Noma et al., 2009; Leidel et al., 2009). Unfortunately, *AtNCS2* failed to complement yeast *Δncs2* mutants. Arabidopsis *ROL5* is quite similar to *NCS6* but also exhibits less conserved regions. AtNCS2 and ROL5 have been shown to interact but the differences in sequence homology of yeast Ncs6p and ROL5 might not allow for an interaction of Ncs6p and AtNCS2 thereby inhibiting tRNA thiolation. Therefore, it will be interesting to check whether complementation of yeast *Δncs2* by *AtNCS2* fails due to the inability of AtNCS2 to bind Ncs6p. To this end, the *Δncs2* mutant can be transformed with *AtNCS2* and *ROL5*, providing both proteins to the system. Furthermore, it will be important to check whether Arabidopsis *ncs2-2* mutants are allowing tRNA thiolation, suggesting that AtNCS2 is indeed involved in this process. Arabidopsis *ncs2-2* mutants exhibit a reduction in primary root lengths. Intriguingly, yeast *Δncs2* has a defect in peusdohyphal growth, suggesting that AtNCS2 and Ncs2p are involved in cell growth (Goehring et al., 2003 b). Considering that tRNA thiolation is required for efficient protein translation, the observed growth phenotype could be explained by a general

alteration of the cellular expression profile (Rogers et al., 1995). In contrast, the *urm1-1 urm2-2* mutant having no detectable level of thiolated tRNAs exhibits no aberrant growth phenotype, making the latter hypothesis unlikely. On the other hand, Ncs2p has also been linked to TOR signaling (Goehring et al., 2003 b). Therefore it can be stated that the observed growth defect of *ncs2-2* could be explained by a general change in the expression profile due to the potential lack of thiolated tRNAS, but could also represent a specific TOR related process. Interestingly, we have shown for ROL5 that indeed both processes can be separated. In future, a similar approach could help to understand whether AtNCS2 is specifically interacting with TOR.

5.6 Apparent homolog of *ROL5* is likely to be a pseudogene

In the Arabidopsis genome one homolog to *ROL5* (*HROL5*) is present. The *hrol5* mutant exhibits no visible mutant phenotype. Because the experimentally determined sequence contains an early stop codon, it can be speculated whether *HROL5* is a pseudogene. Interestingly, the *HROL5* promoter is active. Furthermore, the early stop codon results from an unusual splice site which was not predicted. It is commonly known that under certain environmental conditions differential splicing can occur (Mastrangelo et al., 2012). Furthermore, it has been shown in *D. melanogaster* that alternative splicing is a common process, especially in germ cells, muscle, and the central nervous system (Vernables et al., 2012). Also in Arabidopsis, it has been demonstrated that alternative splicing contributes to the transcriptome and proteome complexity (Zhang and Gassmann, 2011). Therefore, a different splice variant of *HROL5* might be expressed under certain environmental conditions or in different tissues. In future it will be interesting to analyse *HROL5* mRNAs from different tissues and after exposure to different treatments. The finding of differing splice variants would corroborate this theory and would indicate an additional level of regulation of gene expression. At this point no function can be assigned to HROL5, but under altered environmental conditions HROL5 might very well have an important function in plant development.

5.7 Cell wall integrity sensing and TOR – where is the link?

Plant cells need to be able to adapt and maintain the integrity of their cell wall when changes in cell morphology occur, and in response to biotic or abiotic stress. To this end, sensors are thought to monitor small sets of changes in the cell wall structure. These informations need to be integrated in a higher-level regulatory process. In Arabidopsis, the receptor like kinase THESEUS1 and the Wall Associated Kinases (WAKs) represent such sensor systems (Hematy et al., 2007; Ringli, 2010). In a cellulose-deficient mutant background, THESEUS1 has been demonstrated to control the ectopic accumulation of lignin. Furthermore, a complete Arabidopsis transcriptome microarray was performed leading to several potential intracellular targets of THESEUS1 with genes related to cell wall metabolism among them (Hematy et al., 2007). WAKs are involved in the signaling between the extracellular matrix and the cytoplasm. WAK2 has been shown to act on the vacuolar invertase activity and thus influences essential turgor adjustments (Kohorn et al., 2006). Changes in the turgor pressure are known to be the driving force of cell growth (Winship et al., 2011). These examples demonstrate that THESEUS1 and WAKs are able to target intracellular processes regulating certain aspects of cell growth or cell wall dynamics. TOR, at the other hand, represents a likely candidate for a higher-level regulator that monitors several sensing processes and integrates these informations. From TOR research in humans and yeast it is known that TOR regulates a number of processes including several anabolic and catabolic aspects of cellular metabolism as well as memory, aging and cell wall development (Tischmeyer et al., 2003; Martin and Hall, 2005; Hall, 2008; Fuchs et al., 2009). Hence, TOR represents a multi-level key regulator. In this work, we present evidence that the TOR network is not only controlling growth-related events but is also influencing the cell wall architecture. This makes TOR a promising candidate for being an intracellular component of cell wall integrity sensing. In yeast, a link between TOR and cell wall integrity sensing has already been demonstrated in which the connection is established via the small GTPase Rho1 (Levin, 2005; Fuchs

et al., 2009; Tsao et al., 2009). Our work suggests that the small GTPase ROP6 is involved in TOR signaling and physically interacts with ROL5 which is in turn known to be influencing cell wall architecture and TOR. Hence, also in Arabidopsis, small GTPases are involved in establishing the link between TOR signaling and cell wall integrity sensing. Nevertheless, since TOR is known to be a major controller of growth that is influenced by several environmental stimuli and in turn influences several pathways, it is not likely that TOR controls one single aspect of cell wall dynamics. More likely, TOR is controlling general aspects of cell wall development by regulating several downstream processes related to cell wall biosynthesis and cell wall formation. A better understanding of the mechanisms behind cell wall integrity sensing might help to genetically engineer crops with cell walls of more beneficial properties for biofuel production or for human or animal nutrition. In humans, TOR was identified as a potential target for drugs against renal carcinoma, diabetes and tuberous sclerosis (Hudes et al., 2009; Laplante and Sabatini; 2012). Furthermore, inhibition of TOR by rapamycin is used in transplantation medicine (Kawahara et al., 2011). Therefore, a better understanding of the complex network of TOR signaling might also contribute to medical progress in the future.

6 Outlook

The data presented in this work suggest an involvement of ROL5 and ROP6 in TOR signaling as well as in CWI maintenance. A detectable alteration of the cell wall architecture in the *rop6-1* mutant would support the hypothesis that ROP6 indeed has an influence on cell wall development, whereas a changed phosphorylation level of S6K1 in turn would proof that ROP6 is indeed linked to TOR signaling. To obtain a better understanding how TOR is influencing cell growth and cell wall development, it will be helpful to investigate whether also in Arabidopsis, two functional TORC complexes with different functionality exist. This might allow for the identification of downstream pathways linking TOR to cell wall development. The ROL5 interacting proteins AtURM1 and AtURM2 exist as covalently bound protein conjugates. This urmylation process has been described before to

occur upon treatment with ROS in yeast cells (Goehring et al., 2003a; Van der Veen et al., 2011). Considering that in plants ROS are representing a non-enzymatic way of modifying the cell wall structure (Schweikert et al., 2000), this makes it very interesting to investigate whether protein urmylation is influencing cell wall development.

7 Material and Methods

7.1 ROL5

7.1.1 DNA constructs

For localization of *ROL5* in yeast, a cDNA version of *ROL5* was amplified with the primer pair *ROL5_BamHI_*for(ATGGAGGCCAAGAACAAGAAAGCA)/*ROL5_Xba*I_rev(CAATGTGGATCTCTGGATTTCTAA). The fragment was then cloned into *pGEM*-T-easy for sequencing. The C-terminal *GFP-ROL5* fusion was obtained by *Bam*HI digestion and insertion of a *Bam*HI-*GFP* cassette. After *Not*I digestion, the *GFP-ROL5* fusion was cloned into *pFL61* (Minet et al., 1992).

For performing the yeast two-hybrid experiment, cDNA of *ROL5* was amplified with the primer pair *Kpn*I-*At2g44270*-1F(ggtaccatggaggccaagaacaagaaagcag)/SmaI-*At2g44270*-1R(cccgggttagaaatccagagatccacattg). The fragment was then cloned into *pGEM*-T-easy for sequencing. Subsequently, one of the *ROL5* clones was digested with *Kpn*I/*Sac*I and cloned into *pLEXA-N*.

7.2 Δ66_ROL5

7.2.1 DNA constructs

For complementation of the *Δncs6* mutant, a cDNA clone of *Δ66_ROL5* was amplified using the primer pair

*ROL5*_NdeI_F1(GTCCTCAAAAGACCTAAAACC)/*ROL5_Xba*I_rev(CAATGTGGATCTCTGGATTTCTAA). The obtained fragment was cloned into *pGEM*-T-easy for sequencing. Afterwards, a correct clone was digested with *Not*I and cloned into the yeast overexpression vector *pFL61* (Minet et al., 1992).

7.2.2 Yeast complementation

Yeast strains used in this study were obtained from EUROSCARF, Frankfurt, Germany. The wild-type strain is BY4741 with the relevant genotype MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0, and the *Δncs6* strain BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YGL211w::kanMX4. For the rapamycin growth assay, the relevant yeast strains were grown on SD media supplemented with 2.4 nM rapamycin at 30°C for 2-4 days.

7.3 ROP6

7.3.1 DNA constructs

For performing the yeast two-hybrid experiment, cDNA versions of *ROL5* and *ROP6* were amplified with the primer pairs *Kpn*I-*At2g44270*-1F(ggtaccatggaggccaagaacaagaaagcag)/SmaI-*At2g44270*-1R(cccgggttagaaatccagagatccacattg) for *ROL5* and *Bam*HI-*At4g35020*-for (ggatccatgagtgcttcaaggtttatcaa)/ *Xba*I-*At4g35020*-rev(tctagatcagagtatagaacaacctttctg) for *ROP6*. These fragments were then cloned into *pGEM*-T-easy for sequencing. Subsequently, one of the *ROL5* clones was digested with *Kpn*I/*Sac*I and cloned into *pLEXA-N*. The *ROP6* clone was digested with *Bam*HI/*Xba*I and cloned into *pGAD-HA*.

For performing the immuno-precipitation experiment, cDNAs of *ROL5* and *ROP6* were amplified with the primer pairs *ROL5_BamHI*_for(ATGGAGGCCAAGAACAAGAAAGCA)/*Rol5_HA*_rev_STOP(TCTAGATTAAGGTCCTCCCAGGCTGGCATAGTCAGGCACGTCATAAGGATA) for *ROL5* and *ROP6_BamHI*_for(ATGAGTGCTTCAAGGTTTATCAAG)/*ROP6_XbaI*_rev(CAGAAAGGTTGTTCTATACTCTG

A) for *ROP6*. These fragments were then cloned into *pGEM*-T-easy for sequencing. Subsequently, one of the correct *ROL5-HA* clones was digested with *Not*I and cloned into the yeast overexpression vector *pFL61* (Minet et al., 1992). The N-terminal *HIS-ROP6* fusion was produced by digestion with *Bam*HI and insertion of a *Bam*HI-*HIS* linker with the oligonucleotide sequences *HIS*_linker_for (GATCCATGCACCACCACCACCACCACG) / *HIS*_linker_rev (GATCCGTGGTGGTGGTGGTGGTGCATG). The obtained N-terminal *HIS-ROP6* fusion construct was subsequently digested with *Not*I and cloned into *pNEV-E*. For localization of *ROP6* in yeast, cDNA of *ROP6* was amplified with the primer pair *ROP6_BamHI*_for(ATGAGTGCTTCAAGGTTTATCAAG)/*ROP6_XbaI*_rev(CAGAAAGGTTGTTCTATACTCTG A). The fragment was then cloned into *pGEM*-T-easy for sequencing. The N-terminal *GFP-ROP6* fusion was obtained by *Bam*HI digestion and insertion of a *Bam*HI-*GFP* cassette.

7.3.2 Immuno precipitation

A yeast strain containing *HIS_ROP6* and *ROL5_HA* was grown in liquid SD media for two days in a shaker at 37°C. See chapter 7.6.6 for information about yeast growth conditions. Yeast cells were centrifuged for 5 minutes at 3000 rpm at room temperature, the pellet was washed two times with H_2O and resuspended in 300 µl IP buffer (mM Tris, pH 7.5; 150 mM NaCl; 0.1% Nonidet P40; cOmplete Protease Inhibitor Cocktail Tablets (Roche)). All steps were performed at room temperature. Subsequently, yeast cells were vortexed with glass beads for 8 minutes (30 seconds vortex then 30 seconds on ice). After centrifugation for 5 minutes at 13000 rpm at 4°C and subsequent centrifugation of the supernatant for 15 minutes at 13000 rpm at 4°C, the supernatant was transferred to the equilibrated HA-sepharose (Roche). Equilibration of the HA-matrix was done by washing two times with 500 µl equilibration buffer (20 mM Tris, pH 7.5; 0.1 M NaCl; 0.1 mM EDTA). The incubation of the supernatant with the HA-sepharose was done for one hour at 4°C in an overhead shaker. After centrifugation at 4°C for 5 minutes at 13000 rpm, the supernatant was washed two times with wash buffer (20 mM Tris, pH 7.5; 0.1 M NaCl; 0.1 mM EDTA; 0.05% Tween

20). Subsequently one bed volume of 2 times SDS loading buffer (Tris-Cl pH 6.8 25 mM; Urea 9 M; EDTA 1 mM; SDS 1%; β-mercaptoethanol 0.7 M; Glycerol 10 %) was added to the sepharose and boiled for 5 minutes at 95°C. Subsequently, samples were loaded onto a SDS page gel.

7.4 S6K

7.4.1 Phosphorylation assay

For immuno-precipitation of endogenous S6K, approximately 30 seedlings were grown vertically on half-strength MS media and quickly homogenized in 500 µl buffer A (10 mM Tris-Cl pH 7.5; 150 mM NaCl; 1 mM EDTA; 0.05% Nonidet-P40, 2 mM benzamidine, and cOmplete Protease Inhibitor Cocktail Tablets (Roche)) at room temperature. After subsequent centrifugation at 4°C for 20 minutes at 13000 rpm the supernatant was transferred to a fresh tube and 5 µl of p70 S6 kinase α antibody (LabForce sc-8418) was added and incubated over night at 4°C with constant rotation. Then 100 µl of protein A-sepharose beads (Invitrogen) was added and again incubated at 4°C for four hours. The sepharose beads were the washed three times with 1 ml buffer A and two times with 1 ml buffer B (100 mM Tris-Cl pH 7.5; 1 mM NaCl; 0.05% Nonidet-P40; and 0.25% Triton X-100) with centrifugation in-between at 13000 rpm for 1 minute at 4°C. Subsequently, one bed volume SDS loading buffer (Tris-Cl pH 6.8 25 mM; Urea 9 M; EDTA 1 mM; SDS 1%; β-mercaptoethanol 0.7 M; Glycerol 10 %) was added to the supernatant and boiled for 5 minutes at 95°C and loaded onto a SDS page. The phosphorylation of S6K1 was detected with a p-p70 S6K kinase α antibody (LabForce sc-11759) following standard protocols for western blotting.

7.5 NCS2

7.5.1 DNA constructs

For complementation of the yeast *Δncs2* mutant, a cDNA clone of *AtNCS2* was amplified with the primer pair *Xba*I-*At4g35910-1F*(ATGGCTTGTAATTCCTCAGG)/*Bam*HI-*At4g35910*-1R(TTAGACAACCTCTTCATCGT). The fragment was then cloned into *pGEM*-T-easy for sequencing. Subsequently, a correct clone was digested with *Not*I and cloned into the yeast overexpression vector *pFL61* (Minet et al., 1992). For performing the yeast two-hybrid experiment, a cDNA version of *AtNCS2* was amplified with the primer pair *Xba*I-*At4g35910*-1F(tctagaatggcttgtaattcctcagg)/*Bam*HI-*At4g35910*-1R(ggatccttagacaacctcttcatcgt). The fragment was then cloned into *pGEM*-T-easy for sequencing. After digestion with *Bam*HI/*Xba*I the fragment was cloned into *pGAD-HA*.

7.6 General methods

7.6.1 Plant Growth

Seedlings and plants were grown as described by Leiber et al. (2010).

7.6.2 GUS staining procedure

Seedlings or tissue of mature plants containing the *GUS* construct were incubated for 1 – 24 hours in a solution of 1 mg/ml 5-bromo-4chloro-3-indolyl-glucuronid (X-Gluc), 50mM Na-phosphate buffer (pH 7), 10mM EDTA, 3mM DTT. The reaction was stopped and the chlorophyll was removed by incubation with 70% ethanol.

7.6.3 Yeast two-hybrid

The yeast two hybrid experiments were done with the DUALhybrid kit from Dualsystems Biotech.

7.6.4 Microscopy

Epidermal GFP fluorescence was analyzed using a Zeiss Imager Z1 microscope equipped with a Axiocam HRC. GFP fluorescence of yeast cells was analyzed with a Leica DM6000 equipped with a Leica BFC 350FX. Phenotypic observations were performed and GUS staining was analyzed with a Leica LZ M125 stereomicroscope.

7.6.5 tRNA Extraction and Analysis

Arabidopsis seedlings were grown vertically on half-strength MS medium-containing plates for 14 days. Approximately 250 seedlings were used for extraction. The seedlings were grinded in liquid nitrogen and the material was extracted 2 times with 8ml acidic phenol and 0.8ml chloroform and 1 time with 4ml acidic phenol and 0.4ml chloroform. Yeast strains were grown at 30°C in 50ml liquid SD media supplemented with His, Leu, Ade for strains complemented with *pfl61* constructs and His, Leu, Ade and Ura for growth of the *Wt.* The tRNA was extracted 2 times with 4ml acidic phenol and 0.4ml chloroform. Subsequently tRNA was purified with AX100 columns from MACHEREY NAGEL. For analysis the purified tRNA was separated on an acrylamide gel supplemented with N-acryloylamino phenyl mercuric chloride (APM). The method was adapted from Björk et al., (2007).

7.6.6 Yeast growth conditions for complementation or immuno-precipitation experiments

All yeast strains used for complementation or immuno-precipitation experiments had the relevant genotype MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0. Yeast strains transformed with *pFL61* (Minet et al., 1992) were grown in liquid or solid SD media supplemented with adenine, histidine, leucine, and tryptophane. Yeast strains transformed with *pNEV-E* were grown in liquid or solid SD media

supplemented with adenine, uracil, histidine, and tryptophane. All strains grown in liquid or solid SD media were incubated for 2 days at 30°C. Strains incubated in liquid YPAD or YPG media were incubated for 1 day at 30°C and strains incubated on solid YPAD or YPG media were incubated for 2 days at 30°C.

8 References

1. Banerjee, R. *et al.* tRNAs: cellular barcodes for amino acids. *FEBS Lett* **584**, 387-95 (2010).

2. Baumberger, N., Ringli, C. & Keller, B. The chimeric leucine-rich repeat/extensin cell wall protein LRX1 is required for root hair morphogenesis in Arabidopsis thaliana. *Genes Dev* **15**, 1128-39 (2001).

3. Baumberger, N., Steiner, M., Ryser, U., Keller, B. & Ringli, C. Synergistic interaction of the two paralogous Arabidopsis genes LRX1 and LRX2 in cell wall formation during root hair development. *Plant Journal* **35**, 71-81 (2003).

4. Brady, J.D., Sadler, I.H. & Fry, S.C. Pulcherosine, an oxidatively coupled trimer of tyrosine in plant cell walls: its role in cross-link formation. *Phytochemistry* **47**, 349-53 (1998).

5. Breitkreutz, B.J., Stark, C. & Tyers, M. The GRID: the General Repository for Interaction Datasets. *Genome Biol* **4**, R23 (2003).

6. Brisson, L.F., Tenhaken, R. & Lamb, C. Function of Oxidative Cross-Linking of Cell Wall Structural Proteins in Plant Disease Resistance. *Plant Cell* **6**, 1703-1712 (1994).

7. Brutus, A., Sicilia, F., Macone, A., Cervone, F. & De Lorenzo, G. A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. *Proc Natl Acad Sci U S A* **107**, 9452-7 (2010).

8. Caffall, K.H. & Mohnen, D. The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydr Res* **344**, 1879-900 (2009).

9. Carpita, N.C. & Gibeaut, D.M. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J* **3**, 1-30 (1993).

10. Cassab, G.I. Plant cell wall proteins. *Annu Rev Plant Physiol Plant Mol Biol* **49**, 281-309 (1998).

11. Ciechanover, A., Heller, H., Katz-Etzion, R. & Hershko, A. Activation of the heat-stable polypeptide of the ATP-dependent proteolytic system. *Proc Natl Acad Sci U S A* **78**, 761-5 (1981).

12. Cooper, J.B. & Varner, J.E. Insolubilization of hydroxyproline-rich cell wall glycoprotein in aerated carrot root slices. *Biochem Biophys Res Commun* **112**, 161-7 (1983).

13. Cosgrove, D.J. Growth of the plant cell wall. *Nat Rev Mol Cell Biol* **6**, 850-61 (2005).

14. Crowell, E.F., Gonneau, M., Stierhof, Y.D., Hofte, H. & Vernhettes, S. Regulated trafficking of cellulose synthases. *Current Opinion in Plant Biology* **13**, 700-705 (2010).

15. Curtis, M.D. & Grossniklaus, U. A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol* **133**, 462-9 (2003).

16. Denness, L. *et al.* Cell wall damage-induced lignin biosynthesis is regulated by a reactive oxygen species- and jasmonic acid-dependent process in Arabidopsis. *Plant Physiol* **156**, 1364-74 (2011).

17. Denoux, C. *et al.* Activation of defense response pathways by OGs and Flg22 elicitors in Arabidopsis seedlings. *Mol Plant* **1**, 423-45 (2008).

18. Deprost, D. *et al.* The Arabidopsis TOR kinase links plant growth, yield, stress resistance and mRNA translation. *Embo Reports* **8**, 864-870 (2007).

19. Dewez, M. *et al.* The conserved Wobble uridine tRNA thiolase Ctu1-Ctu2 is required to maintain genome integrity. *Proc Natl Acad Sci U S A* **105**, 5459-64 (2008).

20. Doblin, M.S., Kurek, I., Jacob-Wilk, D. & Delmer, D.P. Cellulose biosynthesis in plants: from genes to rosettes. *Plant Cell Physiol* **43**, 1407-20 (2002).

21. Duan, Q., Kita, D., Li, C., Cheung, A.Y. & Wu, H.M. FERONIA receptor-like kinase regulates RHO GTPase signaling of root hair development. *Proc Natl Acad Sci U S A* **107**, 17821-6 (2010).

22. Escobar-Restrepo, J.M. *et al.* The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception. *Science* **317**, 656-60 (2007).

23. Etienne-Manneville, S. & Hall, A. Rho GTPases in cell biology. *Nature* **420**, 629-35 (2002).

24. Ferrari, S. *et al.* Transgenic expression of a fungal endo-polygalacturonase increases plant resistance to pathogens and reduces auxin sensitivity. *Plant Physiol* **146**, 669-81 (2008).

25. Fichtner, L. *et al.* Elongator's toxin-target (TOT) function is nuclear localization sequence dependent and suppressed by post-translational modification. *Mol Microbiol* **49**, 1297-307 (2003).

26. Fry, S.C. Isodityrosine, a new cross-linking amino acid from plant cell-wall glycoprotein. *Biochem J* **204**, 449-55 (1982).

27. Fu, Y. & Yang, Z. Rop GTPase: a master switch of cell polarity development in plants. *Trends Plant Sci* **6**, 545-7 (2001).

28. Fuchs, B.B. & Mylonakis, E. Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. *Eukaryot Cell* **8**, 1616-25 (2009).

29. Furukawa, K., Mizushima, N., Noda, T. & Ohsumi, Y. A protein conjugation system in yeast with homology to biosynthetic enzyme reaction of prokaryotes. *J Biol Chem* **275**, 7462-5 (2000).

30. Gleave, A.P. A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol Biol* **20**, 1203-7 (1992).

31. Goehring, A.S., Rivers, D.M. & Sprague, G.F. Attachment of the ubiquitin-related protein Urm1p to the antioxidant protein Ahp1p. *Eukaryot Cell* **2**, 930-6 (2003).

32. Goehring, A.S., Rivers, D.M. & Sprague, G.F. Urmylation: A ubiquitin-like pathway that functions during invasive growth and budding in yeast. *Molecular Biology of the Cell* **14**, 4329-4341 (2003).

33. Gu, Y., Li, S., Lord, E.M. & Yang, Z. Members of a novel class of Arabidopsis Rho guanine nucleotide exchange factors control Rho GTPase-dependent polar growth. *Plant Cell* **18**, 366-81 (2006).

34. Gutiérrez-Preciado, A., Henkin, T.M., Grundy, F.J., Yanofsky, C. & Merino, E. Biochemical features and functional implications of the RNA-based T-box regulatory mechanism. *Microbiol Mol Biol Rev* **73**, 36-61 (2009).

35. Haas, A.L., Warms, J.V., Hershko, A. & Rose, I.A. Ubiquitin-activating enzyme. Mechanism and role in protein-ubiquitin conjugation. *J Biol Chem* **257**, 2543-8 (1982).

36. Hahn, M.G., Darvill, A.G. & Albersheim, P. Host-Pathogen Interactions : XIX. The endogenous elicitor, a fragment of a plant cell wall polysaccharide that elicits phytoalexin accumulation in soybeans. *Plant Physiol* **68**, 1161-9 (1981).

37. Haiser, H.J., Karginov, F.V., Hannon, G.J. & Elliot, M.A. Developmentally regulated cleavage of tRNAs in the bacterium Streptomyces coelicolor. *Nucleic Acids Res* **36**, 732-41 (2008).

38. Hall, M.N. mTOR-what does it do? *Transplant Proc* **40**, S5-8 (2008).

39. Hall, Q. & Cannon, M.C. The cell wall hydroxyproline-rich glycoprotein RSH is essential for normal embryo development in Arabidopsis. *Plant Cell* **14**, 1161-72 (2002).

40. Hamann, T. & Denness, L. Cell wall integrity maintenance in plants: lessons to be learned from yeast? *Plant Signal Behav* **6**, 1706-9 (2011).

41. Hao, S., Ross-Inta, C.M. & Gietzen, D.W. The sensing of essential amino acid deficiency in the anterior piriform cortex, that requires the uncharged tRNA/GCN2 pathway, is sensitive to wortmannin but not rapamycin. *Pharmacol Biochem Behav* **94**, 333-40 (2010).

42. Hao, S. *et al.* Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. *Science* **307**, 1776-8 (2005).

43. Hayashi, T. & Kaida, R. Functions of xyloglucan in plant cells. *Mol Plant* **4**, 17-24 (2011).

44. Heitman, J., Movva, N.R. & Hall, M.N. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* **253**, 905-9 (1991).

45. Held, M.A. *et al.* Di-isodityrosine is the intermolecular cross-link of isodityrosine-rich extensin analogs cross-linked in vitro. *J Biol Chem* **279**, 55474-82 (2004).

46. Hématy, K. *et al.* A receptor-like kinase mediates the response of Arabidopsis cells to the inhibition of cellulose synthesis. *Curr Biol* **17**, 922-31 (2007).

47. Hershko, A. The ubiquitin system for protein degradation and some of its roles in the control of the cell division cycle. *Cell Death and Differentiation* **12**, 1191-1197 (2005).

48. Hershko, A. & Ciechanover, A. The ubiquitin system. *Annu Rev Biochem* **67**, 425-79 (1998).

49. Howson, R. *et al.* Construction, verification and experimental use of two epitope-tagged collections of budding yeast strains. *Comp Funct Genomics* **6**, 2-16 (2005).

50. Hudes, G.R. *et al.* Clinical trial experience with temsirolimus in patients with advanced renal cell carcinoma. *Semin Oncol* **36 Suppl 3**, S26-36 (2009).

51. Huh, W.K. *et al.* Global analysis of protein localization in budding yeast. *Nature* **425**, 686-91 (2003).

52. Huynh, L.N. *et al.* Linking tRNA localization with activation of nutritional stress responses. *Cell Cycle* **9**, 3112-8 (2010).

53. Jendretzki, A., Wittland, J., Wilk, S., Straede, A. & Heinisch, J.J. How do I begin? Sensing extracellular stress to maintain yeast cell wall integrity. *Eur J Cell Biol* **90**, 740-4 (2011).

54. Jöchl, C. *et al.* Small ncRNA transcriptome analysis from Aspergillus fumigatus suggests a novel mechanism for regulation of protein synthesis. *Nucleic Acids Res* **36**, 2677-89 (2008).

55. John, F., Roffler, S., Wicker, T. & Ringli, C. Plant TOR signaling components. *Plant Signal Behav* **6**, 1700-5 (2011).

56. Kawahara, T., Asthana, S. & Kneteman, N.M. m-TOR inhibitors: what role in liver transplantation? *J Hepatol* **55**, 1441-51 (2011).

57. Klahre, U. & Kost, B. Tobacco RhoGTPase ACTIVATING PROTEIN1 spatially restricts signaling of RAC/Rop to the apex of pollen tubes. *Plant Cell* **18**, 3033-46 (2006).

58. Kost, B. Spatial control of Rho (Rac-Rop) signaling in tip-growing plant cells. *Trends Cell Biol* **18**, 119-27 (2008).

59. Kozminski, K.G. *et al.* Interaction between a Ras and a Rho GTPase couples selection of a growth site to the development of cell polarity in yeast. *Mol Biol Cell* **14**, 4958-70 (2003).

60. Kurowska, M. *et al.* TILLING - a shortcut in functional genomics. *Journal of Applied Genetics* **52**, 371-390 (2011).

61. Laplante, M. & Sabatini, D.M. mTOR signaling in growth control and disease. *Cell* **149**, 274-93 (2012).

62. Lee, K.J., Marcus, S.E. & Knox, J.P. Cell wall biology: perspectives from cell wall imaging. *Mol Plant* **4**, 212-9 (2011).

63. Leiber, R.M. *et al.* The TOR pathway modulates the structure of cell walls in Arabidopsis. *Plant Cell* **22**, 1898-908 (2010).

64. Leidel, S. *et al.* Ubiquitin-related modifier Urm1 acts as a sulphur carrier in thiolation of eukaryotic transfer RNA. *Nature* **458**, 228-U9 (2009).

65. Lin, M. *et al.* The Cdc42 effectors Ste20, Cla4, and Skm1 down-regulate the expression of genes involved in sterol uptake by a mitogen-activated protein kinase-independent pathway. *Mol Biol Cell* **20**, 4826-37 (2009).

66. Loewith, R. *et al.* Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell* **10**, 457-68 (2002).

67. Magnuson, B., Ekim, B. & Fingar, D.C. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signaling networks. *Biochem J* **441**, 1-21 (2012).

68. Mahfouz, M.M., Kim, S., Delauney, A.J. & Verma, D.P.S. Arabidopsis TARGET OF RAPAMYCIN interacts with RAPTOR, which regulates the activity of S6 kinase in response to osmotic stress signals. *Plant Cell* **18**, 477-490 (2006).

69. Martin, D.E. & Hall, M.N. The expanding TOR signaling network. *Curr Opin Cell Biol* **17**, 158-66 (2005).

70. Mastrangelo, A.M., Marone, D., Laidò, G., De Leonardis, A.M. & De Vita, P. Alternative splicing: enhancing ability to cope with stress via transcriptome plasticity. *Plant Sci* **185-186**, 40-9 (2012).

71. Menand, B. *et al.* Expression and disruption of the Arabidopsis TOR (target of rapamycin) gene. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 6422-6427 (2002).

72. Menand, B., Meyer, C. & Robaglia, C. Plant growth and the TOR pathway. *Curr Top Microbiol Immunol* **279**, 97-113 (2004).

73. Micheli, F. Pectin methylesterases: cell wall enzymes with important roles in plant physiology. *Trends Plant Sci* **6**, 414-9 (2001).

74. Minet, M., Dufour, M.E. & Lacroute, F. Cloning and sequencing of a human cDNA coding for dihydroorotate dehydrogenase by complementation of the corresponding yeast mutant. *Gene* **121**, 393-6 (1992).

75. Moreau, M. *et al.* Mutations in the Arabidopsis Homolog of LST8/G beta L, a Partner of the Target of Rapamycin Kinase, Impair Plant Growth, Flowering, and Metabolic Adaptation to Long Days. *Plant Cell* **24**, 463-481 (2012).

76. Moustacas, A.M., Nari, J., Borel, M., Noat, G. & Ricard, J. Pectin methylesterase, metal ions and plant cell-wall extension. The role of metal ions in plant cell-wall extension. *Biochem J* **279 (Pt 2)**, 351-4 (1991).

77. Mucha, E., Fricke, I., Schaefer, A., Wittinghofer, A. & Berken, A. Rho proteins of plants--functional cycle and regulation of cytoskeletal dynamics. *Eur J Cell Biol* **90**, 934-43 (2011).

78. Mucha, E., Hoefle, C., Hückelhoven, R. & Berken, A. RIP3 and AtKinesin-13A - a novel interaction linking Rho proteins of plants to microtubules. *Eur J Cell Biol* **89**, 906-16 (2010).

79. Noma, A., Sakaguchi, Y. & Suzuki, T. Mechanistic characterization of the sulfur-relay system for eukaryotic 2-thiouridine biogenesis at tRNA wobble positions. *Nucleic Acids Res* **37**, 1335-52 (2009).

80. Pedrioli, P.G., Leidel, S. & Hofmann, K. Urm1 at the crossroad of modifications. 'Protein Modifications: Beyond the Usual Suspects' Review Series. *EMBO Rep* **9**, 1196-202 (2008).

81. Perez, P. & Rincón, S.A. Rho GTPases: regulation of cell polarity and growth in yeasts. *Biochem J* **426**, 243-53 (2010).

82. Richman, T.J. *et al.* Analysis of cell-cycle specific localization of the Rdi1p RhoGDI and the structural determinants required for Cdc42p membrane localization and clustering at sites of polarized growth. *Curr Genet* **45**, 339-49 (2004).

83. Ridley, B.L., O'Neill, M.A. & Mohnen, D. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* **57**, 929-67 (2001).

84. Riely, B.K. *et al.* Identification of legume RopGEF gene families and characterization of a Medicago truncatula RopGEF mediating polar growth of root hairs. *Plant J* **65**, 230-43 (2011).

85. Ringli, C. Monitoring the outside: cell wall-sensing mechanisms. *Plant Physiol* **153**, 1445-52 (2010).

86. Ringli, C., Hauf, G. & Keller, B. Hydrophobic interactions of the structural protein GRP1.8 in the cell wall of protoxylem elements. *Plant Physiol* **125**, 673-82 (2001).

87. Robert, S., Mouille, G. & Hofte, H. The mechanism and regulation of cellulose synthesis in primary walls: lessons from cellulose-deficient Arabidopsis mutants. *Cellulose* **11**, 351-364 (2004).

88. Rogers, K.C., Crescenzo, A.T. & Söll, D. Aminoacylation of transfer RNAs with 2-thiouridine derivatives in the wobble position of the anticodon. *Biochimie* **77**, 66-74 (1995).

89. Rubio-Texeira, M. Urmylation controls Nil1p and Gln3p-dependent expression of nitrogen-catabolite repressed genes in Saccharomyces cerevisiae. *FEBS Lett* **581**, 541-50 (2007).

90. Sadava, D. & Chrispeels, M.J. Hydroxyproline-rich cell wall protein (extensin): role in the cessation of elongation in excised pea epicotyls. *Dev Biol* **30**, 49-55 (1973).

91. Schlieker, C.D., Van der Veen, A.G., Damon, J.R., Spooner, E. & Ploegh, H.L. A functional proteomics approach links the ubiquitin-related modifier Urm1 to a tRNA modification pathway. *Proc Natl Acad Sci U S A* **105**, 18255-60 (2008).

92. Schweikert, C., Liszkay, A. & Schopfer, P. Scission of polysaccharides by peroxidase-generated hydroxyl radicals. *Phytochemistry* **53**, 565-70 (2000).

93. Shin, D.H. *et al.* A small GTPase activator protein interacts with cytoplasmic phytochromes in regulating root development. *J Biol Chem* **285**, 32151-9 (2010).

94. Shirsat, A.H., Wieczorek, D. & Kozbial, P. A gene for Brassica napus extensin is differentially expressed on wounding. *Plant Mol Biol* **30**, 1291-300 (1996).

95. Sormani, R. *et al.* Saccharomyces cerevisiae FKBP12 binds Arabidopsis thaliana TOR and its expression in plants leads to rapamycin susceptibility. *Bmc Plant Biology* **7**(2007).

96. Soulard, A., Cohen, A. & Hall, M.N. TOR signaling in invertebrates. *Current Opinion in Cell Biology* **21**, 825-836 (2009).

97. Stintzi, A. & Browse, J. The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. *Proc Natl Acad Sci U S A* **97**, 10625-30 (2000).

98. Thomas, C., Fricke, I., Weyand, M. & Berken, A. 3D structure of a binary ROP-PRONE complex: the final intermediate for a complete set of molecular snapshots of the RopGEF reaction. *Biol Chem* **390**, 427-35 (2009).

99. Thompson, D.M., Lu, C., Green, P.J. & Parker, R. tRNA cleavage is a conserved response to oxidative stress in eukaryotes. *RNA* **14**, 2095-103 (2008).

100. Thompson, D.M. & Parker, R. The RNase Rny1p cleaves tRNAs and promotes cell death during oxidative stress in Saccharomyces cerevisiae. *J Cell Biol* **185**, 43-50 (2009).

101. Tischmeyer, W. *et al.* Rapamycin-sensitive signaling in long-term consolidation of auditory cortex-dependent memory. *Eur J Neurosci* **18**, 942-50 (2003).

102. Tsao, C.C., Chen, Y.T. & Lan, C.Y. A small G protein Rhb1 and a GTPase-activating protein Tsc2 involved in nitrogen starvation-induced morphogenesis and cell wall integrity of Candida albicans. *Fungal Genet Biol* **46**, 126-36 (2009).

103. Van der Veen, A.G. *et al.* Role of the ubiquitin-like protein Urm1 as a noncanonical lysine-directed protein modifier. *Proc Natl Acad Sci U S A* **108**, 1763-70 (2011).

104. Wang, F., Liu, M., Qiu, R. & Ji, C. The dual role of ubiquitin-like protein Urm1 as a protein modifier and sulfur carrier. *Protein Cell* **2**, 612-9 (2011).

105. Wang, G.D. *et al.* The Diverse Roles of Extracellular Leucine-rich Repeat-containing Receptor-like Proteins in Plants. *Critical Reviews in Plant Sciences* **29**, 285-299 (2010).

106. Wang, J.B., Sonn, R., Tekletsadik, Y.K., Samorodnitsky, D. & Osman, M.A. IQGAP1 regulates cell proliferation through a novel CDC42-mTOR pathway. *J Cell Sci* **122**, 2024-33 (2009).

107. Willats, W.G., McCartney, L., Mackie, W. & Knox, J.P. Pectin: cell biology and prospects for functional analysis. *Plant Mol Biol* **47**, 9-27 (2001).

108. Winship, L.J., Obermeyer, G., Geitmann, A. & Hepler, P.K. Pollen tubes and the physical world. *Trends Plant Sci* **16**, 353-5 (2011).

109. Wu, N. *et al.* Natural rules for Arabidopsis thaliana pre-mRNA splicing site selection. *Central European Journal of Biology* **7**, 620-625 (2012).

110. Wullschleger, S., Loewith, R. & Hall, M.N. TOR signaling in growth and metabolism. *Cell* **127**, 5-19 (2006).

111. Xiong, Y. & Sheen, J. Rapamycin and glucose-target of rapamycin (TOR) protein signaling in plants. *J Biol Chem* **287**, 2836-42 (2012).

112. Xu, T. *et al.* Cell surface- and rho GTPase-based auxin signaling controls cellular interdigitation in Arabidopsis. *Cell* **143**, 99-110 (2010).

113. Yan, G., Lai, Y. & Jiang, Y. The TOR Complex 1 Is a Direct Target of Rho1 GTPase. *Mol Cell* **45**, 743-53 (2012).

114. Yang, Z. Small GTPases: versatile signaling switches in plants. *Plant Cell* **14 Suppl**, S375-88 (2002).

115. Yang, Z. & Watson, J.C. Molecular cloning and characterization of rho, a ras-related small GTP-binding protein from the garden pea. *Proc Natl Acad Sci U S A* **90**, 8732-6 (1993).

116. Zhang, X.C. & Gassmann, W. Quantifying alternatively spliced mRNA via capillary electrophoresis. *Methods Mol Biol* **712**, 69-77 (2011).

117. Zhang, X., Ren, Y. & Zhao, J. Roles of extensins in cotyledon primordium formation and shoot apical meristem activity in Nicotiana tabacum. *J Exp Bot* **59**, 4045-58 (2008).

118. Ziman, M. *et al.* Subcellular localization of Cdc42p, a Saccharomyces cerevisiae GTP-binding protein involved in the control of cell polarity. *Mol Biol Cell* **4**, 1307-16 (1993).

9 Abbreviations

APM	[(N-acryloylamino) phenyl] mercuric chloride
AX	Arabinoxylan
AGP	arabinogalactan protein
aaRS	aminoacyl-tRNA synthetase
bp	base pair
CELS	cellulose synthesis complex
CESA	cellulose synthesis complex gene family
CWI	cell wall integrity
DTT	1,4-dithio-DL-threitol
EDTA	disodium ethylene diamine tetraacetate
GAX	Glucuronoarabinoxylans
GX	Glucuronoxylan
GDI	GDP dissociation inhibitor
GRP	glycine rich proteins
GEF	guanine nucleotide exchange factor
GAP	GTPase activating protein
HRGP	hydroxyproline-rich glycoproteins
HG	Homogalacturonan
HYP	Hydroxyproline
GAL	Galactose
LRR	leucine-rich repeat
LRX	leucine-rich extensin
MS	Murashige and Skoog
MCM5U	5-methoxycarbonylmethyluridine
mLST8	target of rapamycin complex subunit
OGA	Oligogalacturonides
PCR	polymerase chain reaction
PME	pectin methylesterase
RGI	rhamnogalacturonan I

RGII	rhamnogalacturonan II
ROP	Rho of plants
RHO	RAS-like proteins
RT-PCR	reverse transcription polymerase chin reaction
raptor	regulatory-associated protein of mTOR
rictor	rapamycin-insensitive companion of mTOR
SER	Serine
ROL	repressor of lrx1
TOR	target of rapamycin
TORC1	CREB-regulated transcription coactivator 1
TORC2	CREB-regulated transcription coactivator 2
tRNA	transer ribonucleic acid
URM	ubiquitin related modifier
UBL	ubiquitin like protein
UDP-Glc	uridinediphosphate glucose
WAK	walls-associated kinase
XGLUC	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt
xylp	Xylopyranose

10 Appendix

ROL5 coding sequence

ATGGAGGCCAAGAACAAGAAAGCAGTAGCCTCCCGTCTCTGCTGCTTATGCAACCTGAGA

CGTCCCGTCCTCAAAAGACCTAAAACCCTTCAACAGGTAACTTTTCAATTTTTTTTATTT

CTCTTTTCTCGTAGAATCTCGTTGGGTTTTAGCTAACATATTAAGCAGAAGCTTGGAACA

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ATGGAGGCCAAGAACAAGAAAGCAG	**KpnI-At2g44270-1F**
GTCCTCAAAAGACCTAAAACC	**ROL5_NdeI_F1**
CAATGTGGATCTCTGGATTTCTAA	**ROL5_XbaI_rev**
AAACAATGTGGATCTCTGGATTTCTAA	**SmaI-At2g44270-1R**

ROP6 coding sequence

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CAGAAAGGTTGTTCTATACTCTGA	**ROP6_XbaI_rev**
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GATCCGTGGTGGTGGTGGTGGTGCATG	**Bam_HIS_linker_rev**

NCS2 genomic sequence

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URM1 genomic sequence

CCAGCTCTATTTTGCGACTCGGATTGGTTAGAATCTTTCTCCTCATTAGGTGTTTCCCCT

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ATGCAATTAACTCTTGAATTCG	**URM1_Xba1_for**
TTCCACTTTGCATGGTGGATAA	**URM1_BamH1_rev**
CAATTAACTCTTGAATT	**URM1_HA_for**
TCACACTTCCATGAACACAAAA	**URM1_prom_450**
AATCGACAGAGAATCAGAACAT	**URM1_prom_1000**
ATGCAATTAACTCTTGAATTCGGGTAC	**UMR1_HA_gen_f_Xho**

TGATATTAGAGTATTTATCAATTTTAAGTGTCCTTTC **UMR1_HA_gen_ r_Xba**

URM2 genomic sequence

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TGGTCTTTTTTTATTCGGCGGATTTGTGGCGGAAACTTTCATTCTTCTATGTGATATCTA

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ACATGAGATATAGCCACAATTGAAAACTGTCGAATTATATGGTCTTTTTTTATTCGGCGG

AATCGTGGCGGAAACTTTTATTCTTCTATGTGATATCTACGGTTTAAATGATGACGCGGA

AAGATCTCGTTCTCGTGCGCTATCTCCACGTGTTGAATGTCACACGCCGGAGAATGTCTA

CTGTTTTGATTCTTGCCTAAGAAAACAAACTGTTGTGATTCTGTTTCAATAAGCGTTTGG

GTATTTTTAAGAAGGGCTTTGATATTTGGTGATCATGTTGCCTAACTTTAAGGGCCCAAA

CCAAGAAAACGAAAACGACGTCGTATGGAGTTTTGCGTGAACTCAAAGCGGAGATCCTAT

CGGAGAGAATCGGAGAAGAAGAAAAAGAAGAAGAAGGGTTTTGTTACCGCAAAACTTCAT

TGAAG^{ATG}CAATTTACTCTTGAGTTCGGGTACACTATTACTCGTTTGTGTCATTTGCCCA

CTCTCATAGTGGTTTATATTTTGATTTCATTTGATTGACTCTCAAATGTCAATGCCTGTT

TATTGGTCGGGAAAAAACTGAAAACTTAGTGCTTTAGATTATGGTTTCTCTATTTGCTTG

GGATATTATAATGCACAGTTTGTAGCATAAGCCTATCTTTGTTTTGCTTATTCAGTGCTA

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GTCATCATCAGATCAATTGGTAGTGAATGAGAGTTTCGTATTGGTATTAACATGAAAATA

TGTGCTTGTTGATCTCCTGCAGTGGAGGTTTAGAATTGCTCTGTGACTCCGTAAAGATTC

ATAAAGTTAACATCAACTTACTCAATGATTCTGATATCGTAAGCTTCTCTCTTTTGATGA

TTTTTAAATACCATTGAGTTTATTATAGTCATGTAAAACTTCTCACTGGTGTTTCATTCA

GTACAGTTGACAATGAAGGATTTGCTTTCATGGGTTCGTACCAATTTGATCAAGGAAAGG

CCTGAAATGTTCATGAAAGGCGATACCGTGTAAAGCTTCTCTCTTTTCTTTCCTTGATTT

CCAGCAAGAACTCTGTTTTTAGTGTTTAATAACAATGGTGGTTTGGAATTCATAAACGCA

GGAGGCCTGGAGTTCTCGTGCTGGTTAATGACTGCGATTGGGAACTTAGTGGTCAGCTCG

ATACAACATTAGAAGATAAAGATGTTATAGTTTTCATTTCGACTCTGCACGGTGGA^{TGA}A

TATTGAAATAGCAGAGCCTTGATCATTTTGATCGTTTCCAACTACTTGTGATGAACATTC

CACAAACCTTTGTAATCTCTGGCTCATTGTTATTACTTTGAGTTTATATTTGCAACCACA

TCATTCGTAATTGAGGAGTTTTGATGTTATGAATCTTAGCTACCAGAATCAGATTCACCT

CATGGTTGCTTTAATGCAGTGTGCCTTATGGATGTAGTATTAGTATATTATTGAAATTCT

CTCTACATACTGAAAAATGTTTGGAAACAAGACTTACATAAGAAAAAGTTAAAAAAAAAA

AAAAAAAAAAAACTAGACAAATTTAGGATGTTTTTTGTTTGTTTATTTA

ATGCAATTTACTCTTGAGTTCGGGTACACTATTAC **URM2_HA_ge_f_Xho**

TCATCCACCGTGCAGAGTCGAAATGAAAACTATA **UMR2_HA_gen_ r_Xba**

URM2 coding sequence

ATGCAATTTACTCTTGAGTTCGGTGGAGGTTTAGAATTGCTCTGTGACTCCGTAAAGATT

CATAAAGTTAACATCAACTTACTCAATGATTCTGATATCTTGACAATGAAGGATTTGCTT

TCATGGGTTCGTACCAATTTGATCAAGGAAAGGCCTGAAATGTTCATGAAAGGCGATACC

GTGAGGCCTGGAGTTCTCGTGCTGGTTAATGACTGCGATTGGGAACTTAGTGGTCAGCTC

GATACAACATTAGAAGATAAAGATGTTATAGTTTTCATTTCGACTCTGCACGGTGGATGA

ATGCAATTTACTCTTGAGTTCGGT	**URM2_for**
TCATCCACCGTGCAGAGTCGAAAT	**URM2_rev**

11 Curriculum vitae

JOHN

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Education

7/2000	A-levels Lise-Meitner-Gymnasium, Germany
4/2008	Diploma in Agricultural Biology (equivalent to the master) University of Hohenheim, Germany **Biotechnology, General Virology, Medical Microbiology** **Diploma Thesis: "Cloning and Sequence analysis of the Maracuja Mosaic Virus"**

Employment

11/2007 – 01/2008	Bayer CropScience, Frankfurt **Internship** **"Development of an invitro herbicide resistance screening assay"**
09/2005 – 12/2005	Selecta Klemm GmbH&Co KG, Stuttgart **Internship** **"classical and invitro breeding methods"**

Ph.D. Thesis

10/08 – 09/12 **"The TOR Pathway, a Central Relay Linking Cell Growth and Cell Wall Dynamics in *Arabidopsis thaliana*"**

Supervisor: PD Dr. Christoph Ringli
University of Zurich, Switzerland

Publications

Florian John, Ruth-Maria Leiber, et al (2010).

The TOR Pathway Modulates the Structure of Cell Walls in Arabidopsis. The Plant Cell

Florian John et al. (2011).

Plant TOR signaling components. Plant signaling&behavior

Florian John et al. (2012)

Ubiquitin-related modifiers of Arabidopsis function in tRNA modification and protein conjugation.

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